A Study of *Mycoplasma suis* Pathomechanisms Leading to Immune Modulation and Hemorrhagic Diathesis

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“ Life in the microbial world is no picnic. Hard molecular rain falls incessantly from all sides, nutrients are scarce and unpredictable, experienced competitors lurk at every turn. Faced with continual life and death decisions, survival depends on cunning and quick reflexes.” Parkinson, 1993.

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1 Summary

Hemotrophic mycoplasmas (HMs) are red blood cell (RBC) parasites that cause acute and chronic anemia in a variety of vertebrates. A hallmark of this group of cell wall-less, pleomorphic bacteria is their small genome and size. HMs depend on metabolic nutrients of their host for proliferation. As a consequence, a splenectomized animal model is necessary for HM propagation.

Interestingly, reports on human infections have increased significantly, thereby indicating a zoonotic potential of hemotrophic mycoplasmas. Several HMs have been reported to play a role in immune mediated diseases for example, the pig specific *Mycoplasma suis* was shown to play a role in the development of systemic lupus erythematosus (SLE).

M. suis infections are characterized by the lifelong persistence of the agent and development of pathological alterations e.g. hemorrhagic diathesis. The modulation of the host's immune response helps *M. suis* to avoid destruction and to establish persistent infections. To date, detailed studies on immunomodulatory activity of *M. suis* are lacking due to the constraints linked to the animal model and the inability to cultivate *M. suis in vitro*.

So far it is only known that *M. suis* alters the *in vitro* immune response of lymphocytes from *M. suis* infected pigs. Firstly it inhibits a blastogenic response of T-lymphocytes. Secondly, it induces a polyclonal activation of B-lymphocytes that is characterized by the production of autoreactive IgM and IgG antibodies. However, mechanisms mediating immune evasion have hitherto not been elucidated. This study focuses on the identification of immune system components that are targeted by *M. suis* and the documentation of infection induced pathologies.

The interaction of *M. suis* with peripheral blood mononuclear cells (PBMCs) was analyzed *in vivo* and *in vitro*. In 12% of the PBMCs *M. suis* appeared as single or cluster of cells either on the inside or attached to their surface. The total PBMC count was considerably decreased and at the same time a widespread clotting of the blood occurred upon *M. suis* infection.

In the present study three key events that characterize the immune modulation by *M. suis* were evidenced. Firstly, in the early phase of interaction *M. suis* induces a T-lymphocyte-independent

polyclonal activation of naive B-lymphocytes. Secondly, *M. suis* inhibits the T-helper cell mediated immune response via premature activation of T-regulatory cells. The suppression of the T-helper cell mediated immune response by T-regulatory cells derived IL-10, represents a mechanism responsible for the development of tolerance to *M. suis* antigens. Thirdly, macrophage derived IL-12 was down-regulated upon stimulation with *M. suis*. The down-regulation of IL-12 affects the development of type 1 immune response of T-helper cell (Th1) and the elimination of intracellular pathogens.

In *M. suis* infected pigs intravasal coagulation and subsequent consumption coagulopathy were reported previously as mechanisms responsible for hemorrhagic diathesis and coagulation dysfunction. However they cannot fully explain the cascade of events responsible for the pathology. Therefore, the focus of the present study was to analyze the contribution of *M. suis*-endothelial cells (ECs) interaction to the subsequent hemorrhagic diathesis. The activation of ECs upon *M. suis* interaction *in vitro* was evidenced by flow cytometry analysis. The *in vivo* dysfunctional activation and widespread damage of ECs was documented using light and electron microscopy. Dysfunctional activation and disturbance of protective function of endothelial cells (ECs) upon *M. suis* infection was evidenced. Furthermore, *M. suis* single cells and microcolonies attached to the endothelium resembling biofilm formation were documented by electron microscopic analysis. Biofilm formation usually represents a persistence mechanism of bacteria.

In conclusion this thesis provides first evidence that *M. suis* was not only found attached to erythrocytes and free in plasma but also attached to ECs and PBMCs. *M. suis* induced leukopenia is attributed to the coagulopathy and it represents immune system impairment and supports the persistence of the agent. *M. suis* inhibits T-helper cell response, thereby preventing the generation of *M. suis* specific cellular immune response. Most probably, *M. suis* impairs macrophage function as indicated by the down-regulation of IL-12. The T-helper cell independent B-lymphocyte polyclonal activation by *M. suis* leads to the production of autoreactive antibodies, resulting in the autoimmunity. Dysfunctional activation and disturbance of protective function of endothelial cells upon *M. suis* infection leads to hemorrhagic diathesis and organ failure. Altogether we were able to identify key components of the immune response affected by *M. suis* in the early phase of infection which represents a crucial phase for the generation of a protective immune response.

2 Zusammenfassung

Hämotrophe Mykoplasmen (HM) infizieren die roten Blutzellen verschiedener Vertebraten und verursachen dabei akute und chronische Anämien. Diese zellwandlose und pleomorphe Gruppe von Bakterien besitzt ein sehr kleines Genom. Die bislang *in vitro* unkultivierbaren HM sind für ihre Vermehrung auf die Zufuhr von Wirtsmetaboliten angewiesen und können nur im splenektomierten Tiermodell vermehrt werden.

Berichte über humane HM-Infektionen sind in den letzten Jahren signifikant angestiegen und weist auf ein zoonotisches Potential dieser Bakterien hin. Verschiedene HM-Vertreter spielen eine Rolle in immunvermittelten Krankheiten. So wurde z.B. beschrieben, dass *Mycoplasma suis,* das klassischerweise spezifisch Schweine infiziert, eine Rolle in der Entwicklung des Systemischen Lupus Erythematosus (SLE) beim Menschen spielen kann.

Infektionen des Schweines mit *M. suis* sind durch eine lebenslange Persistenz des Erregers und durch die Entwicklung pathologischer Veränderungen, wie z.B. hämorrhagischer Diathese, charakterisiert. Durch die Modulation der Immunantwort des Wirtes wird die *M. suis*-spezifische Immunabwehr beeinflusst, was wiederum eine Persistenz des Erregers ermöglicht. Aufgrund der Einschränkungen im Zusammenhang mit dem Tiermodell und dem Fehlen eines *in vitro* Kultivierungssystems für *M. suis*, gibt es keine detaillierten Studien über immunmodulatorische Aktivitäten dieses Bakteriums.

Es ist bekannt, dass *M. suis* die Immunantwort von Lymphozyten aus *M. suis*-infizierten Schweinen *in vitro* beeinflusst. Einerseits wird die Blastogenese der T-Lymphozyten inhibiert und andererseits wird eine polyklonale Aktivierung der B-Lymphozyten induziert, welche zur Produktion von autoreaktiven IgM und IgG Antikörpern führt. Die Mechanismen, die für die Immunmodulation und die Immunevasion verantwortlich sind, wurden bislang nicht aufgeklärt. Die vorliegende Arbeit fokussiert daher auf die Identifizierung der Immunsystem-Komponenten, die von *M. suis* beeinflusst und moduliert werden, sowie auf der Aufklärung von pathologischen Veränderungen, die durch eine *M. suis*-Infektion verursacht werden.

Die Interaktion von *M. suis* mit mononukleären Zellen des peripheren Blutes (PBMCs) wurde *in vivo* und *in vitro* untersucht. Bei 12% der PBMCs konnte *M. suis* als einzelne Zelle oder in Form von

Clustern auf oder in Zellen *in vivo* nachgewiesen werden. Die Gesamtzahl der PBMCs in infizierten Schweinen war deutlich vermindert und gleichzeitig trat eine erhöhte Blutgerinnung auf.

Insgesamt wurden durch die vorgelegte Studie drei für eine Immunmodulation durch *M. suis* entscheidende Charakteristika, nachgewiesen:

1. In der frühen Phase der Interaktion induziert *M. suis* eine T-Lymphozyten-unabhängige polyklonale Aktivierung der B-Lymphozyten.

2. *M. suis* inhibiert die T-Helferzellen-vermittelte Immunantwort durch frühzeitige Aktivierung der regulatorischen T-Zellen, welche IL-10 produzieren. Die Hemmung der durch T-Helferzellen induzierten Immunantwort durch IL-10 repräsentiert einen Mechanismus, der für die Entwicklung der Toleranz gegenüber *M. suis*-Antigenen verantwortlich ist.

3. IL-12, das durch Makrophagen produziert wird, wurde nach der Interaktion mit *M. suis* herunterreguliert. Diese Herabregulation von IL-12 beeinflusst die Entwicklung der T-Helferzellen-vermittelten Immunantwort des Typs 1 (Th1) und somit die Eliminierung von intrazellulären Pathogenen.

Als Ursachen für die während *M. suis*-Infektionen beobachteten Gerinnungsstörungen und hämorrhagischen Diathesen wurden in einer früheren Studie eine intravasale Gerinnung mit anschliessender Verbrauchskoagulopathie identifiziert. Jedoch konnten durch diese Pathomechanismen nicht alle *M. suis*-induzierten Veränderungen erklärt werden. Das Ziel dieser Studie war daher, die Interaktion von *M. suis* mit Endothelzellen (EZ) im Hinblick auf die Entstehung der hämorrhagischen Diathese zu analysieren.

Licht- und Elektronenmikroskopisch konnten in infizierten Schweinen grossflächige Veränderungen und Zerstörungen des Endothels nachewiesen werden. Ausserdem wurde mittels Durchflusszytometrie eine massive Aktivierung von EZ *in vitro* demonstriert werden

Diese grossflächige und massive Aktivierung sowie die pathologischen Veränderungen der EZ sind aufgrund der Studienergebnisse ursächlich für Hämorrhagien und Dysfunktionen der Organe. Zudem konnten mittels elektronenmikroskopischer Analyse einzelne *M. suis*-Zellen und biofilmähnliche *M. suis*-Mikrokolonien in enger Assoziation mit dem Endothelium dokumentiert werden. Die Bildung von Biofilmen ist als Persistenzmechanismus bekannt.

In dieser Arbeit wird zum ersten Mal gezeigt, dass *M. suis* nicht nur, wie bisher angenommen, mit Erythrozyten interagiert oder frei im Plasma zu finden ist, sondern auch in enger Assoziation mit Endothelzellen und PMBCs. Die *M. suis*-induzierte Leukopenie wird der Koagulopathie zugeschrieben und trägt zu einer Schwächung des Immunsystems bei, was wiederum die Erregerpersistenz begünstigt.

M. suis inhibiert die durch T-Helferzellen vermittelte Immunantwort wodurch die Entwicklung einer zellulären, *M. suis*-spezifischen Immunantwort verhindert wird. Die Herunterregulation von IL-12 weist zudem auf eine Inhibierung der Makrophagenfunktion hin. Die T-Helferzellen-unabhängige polyklonale Aktivierung der B-Lymphozyten durch *M. suis* führt zur Produktion autoreaktiver Antikörper. Die dysfunktionale Aktivierung und die Beeinträchtigung der schützenden Funktion der EZ durch die *M. suis*-Infektion führen zu hämorrhagischer Diathese und Organfehlern.

In dieser Arbeit konnte gezeigt werden, dass drei Komponenten der Immunantwort in der frühen Phase der Interaktion von *M. suis* beeinflusst werden. Die frühe Phase der Interaktion ist entscheidend für die Entwicklung einer schützenden Immunantwort.

3 Introduction

3.1 The genus *Mycoplasma*

Mycoplasmas are phenotypically distinguishable from all other bacteria by the lack of cell wall and the minute size and genome [1, 2]. Because of the lack of the cell wall mycoplasmas are clustered in the class of Mollicutes (lat. – soft), which is classified within the phylum of Tenericutes and consists of more than 250 species [3, 4]. The genus *Mycoplasma* comprises pathogens of mammals, birds, reptiles, arthropods, plants, and fish [2]. Infections with mycoplasmas are associated with retroviral and autoimmune diseases [5, 6]. Mycoplasmas evade or suppress their host defense mechanisms either directly by invading and destroying immune cells (e.g. antibody presenting cells) or indirectly by inducing autoimmunity, mitogenicity, modulation of cytokine expression levels, etc. [1].

Mycoplasmas are species characterized by an extremely A-T rich chromosome and the smallest genome of any independently replicating organism [7-11]. Their uniqueness comes in addition to the small chromosome by the exceptional codon usage. In mycoplasmas, the stop codon, TGA is used as tryptophan encoding codon instead [2, 12].

The genera of *Eperythrozoon* and *Haemobartonella* were previously classified within the order *Rickettsiales* as member of the family *Anaplasmataceae.* Based on the phenotypical and phylgenetical characteristics, these two genera were found to be more closely related to the class *Mollicutes* [13]. Therefore they have been reclassified to the genus *Mycoplasma* (pneumoniae group) building the distinct new cluster of hemotrophic mycoplasmas [13, 14]. The classification was based on RNA sequence analysis and phenotypic characteristics [1, 3, 14, 15].

3.2 Hemotrophic mycoplasma

The Hemotrophic mycoplasma (HM) cluster consists of pleomorphic red blood cell (RBC) parasites that infect a wide range of animals and cause hemolytic anemia [13, 16, 17].

A common feature of HMs is their ability to attach to the surface of host RBC and parasitize them [12, 17, 18]. They have a variable morphology appearing as rings, coccoids or short rods of 0.3 to 3 microns in diameter [16, 19]. Hence, cell wall targeting antimicrobial agents do not eliminate HMs, as

they do not have a cell wall [1, 13]. The HMs are not cultivable *in vitro* so far, and as a consequence thereof little is known about their pathogenic mechanisms [13, 20]. This group of bacteria is highly adapted to their host. Their adaptation is characterized by complex nutritional requirements, induction of persistent infection, and modulation of the host immune responsiveness [13, 16, 20, 21]. Nutritional dependence of HMs on complex host cell metabolism is responsible for the lack of *in vitro* HM cultivation system [16].

In 1928 Dr. Schilling identified, for the first time, mouse- and dog-specific HMs (*E. coccoides, H. canis*) [22], since then species-specific HMs have been described in the blood of several animals (cat, mouse, rat, sheep, goat, cattle, pig, opossum, llama, horse, and squirrel monkey) [13, 23]. Recently, a new human-specific species was described which presented clinical signs similar to those characterizing HM infections in other vertebrates [24].

Infections with pig specific Hemotrophic *Mycoplasma suis* (*M. suis*) are common among livestock and cause considerable economic losses [25]. In 2008 a report evaluating the prevalence of *M. suis* found up to to 40 % infection in German pig farms [25].

The zoonotic potential of HM has been reported several times in the past [13, 16, 26, 27]. *M. suis* was found in 49 % of tested swine-farm workers in Shanghai [28]. Other zoonotic HM infections reported include an HIV positive patient infected with *Mycoplasma haemofelis* in Brazil and a veterinarian was infected with *Mycoplasma ovis* in California [29, 30].

It is noteworthy that HM - infections are associated with autoimmune and retroviral diseases [16].

3.3 *Mycoplasma suis* (*M. suis*)

The porcine pathogen *M. suis* is able to adhere to the surface of RBCs and even to invade them in an endocytosis-like process [13, 18]. *M. suis* appears in coccoid, discoid, and ring form of approximately 200 nm in diameter in association with RBCs or free floating in the plasma [31].The close contact between RBCs and *M. suis* is characterized by the presence of a 30 nm lucent zone and fine fibrillar attachments between the two interacting partners [13].

Little is known about microbial, virulence and persistence mechanisms of *M. suis* since an *in vitro* cultivation system has not been established [12, 20, 32, 33]. Therefore, the splenectomized pig, a well-established animal model, is used to propagate *M. suis* [34]. In this *in vivo* model all relevant clinical and microbiological parameters of a natural infection can be simulated [35]. These include: hypoglycemia, immune-hemolytic anemia, autoreactive antibody production, cyanoses, immune modulations, pathogen persistence, and circulatory disturbances [13, 36].

M. suis, like other members of HMs, has a very small genome (709 Kb) for a self-replicating organism [21]. The small genomic size indicates the loss of genetic information for different biosynthesis pathways and explains *M. suis* requirements for host nutritional molecules [20, 21, 37].

The recent in silico genomic analysis predicts *M. suis* ability to catabolize glucose and synthesize purines from hypoxanthine [37], which is in line with the results from the proteome analysis [20]. Like other mollicutes, *M. suis* is not capable of *de novo* synthesis of nucleotides and coenzymes and must therefore import them from the host [20, 37]. The lipid metabolism is also restricted according to genomic and proteomic analysis [20, 37]. These results further support the suggested HM evasion strategy, which consists of HMs coating itself with parts of the host membrane [31].

3.4 Infectious anemia in pig (IAP)

As a consequence of complex nutritional dependence on the host, *M. suis* is bound to a parasitic lifestyle and represents the causative agent of either acute or chronic IAP [13, 38]. The acute course of *M. suis* induced hemolytic anemia is severe and leads to death, whereas mild anemia, ill thrift, infertility, and immune suppression are features of the chronic course of the disease [13, 31, 36]. Immune suppressed animals and suckling piglets suffer acute course of hemolytic anemia [37]. The chronic course of the infection signed for instance by a higher susceptibility to infections of the gastrointestinal and the respiratory tract has a great economic impact in livestock production [13, 36]. Clinical patterns of the chronic course of the disease result from the ability of *M. suis* to persist life-long and cause modulation of host immune system including autoimmunity induction [33, 39].

Host immune response to *M. suis*

Alteration and deformation of RBCs during *M. suis* infection induce the production of anti RBC autoantibodies that are responsible for clinical signs e.g. acrocyanosis and pallor [33, 36, 40-42].

Proteome and immunome analysis performed by Hoelzle and co-workers identified *M. suis* antigens expressed during IAP. They were targeted by porcine IgGs [12, 40, 41, 43-45]. These immunogens were identified as two heat shock proteins (DnaK, GroEL), two glycolysis enzymes (enolase, pyruvate dehydrogenase), one RNA helicase and an actin-analogous protein [40]. Further analysis characterized the following 3 antigens as moonlighting proteins: HspA1 (Heat shock protein A1), MSG1 (*M. suis* GAPDH-like protein 1), and α-Enolases. The first has a chaperonin and cell adhesion function. The last two have glycolytic and cell adhesion function [12, 40, 45].

In the acute phase of immune mediated hemolytic anemia an increase of autoreactive IgG antibodies and a decrease of *M. suis*-specific antibodies was observed [41]. Further analysis identified autoreactive antibodies of IgG isotype directed to host actin [13, 39]. In addition, immunization with recombinant MSG1 was shown to induce production of auto-actin- antibodies in pigs, indicating a role of MSG1 in induction of autoimmunity by molecular mimicry [39].

M. suis infections affects both B- and T-lymphocyte response as determined *in vitro* by ^{3}(H)thymidine incorporation assay [36]. The polyclonal activation of T-lymphocytes from *M. suis* infected pig was inhibited *in vitro*. Against it, B-lymphocytes were activated in a polyclonal manner *in vitro*.

3.5 Immune system

The immune system is composed of organs and cells that collaborate to protect the multicellular organism from invading pathogens [46, 47]. Immunity refers to the condition in which an organism is protected from foreign organisms [47]. In vertebrates two types of immune systems operate cooperatively to protect the organism: the acquired immune system, and the phylogenetically ancient innate immune system [46, 48].

The innate immune system is the first line of the defense with limited specificity that allows recognition of molecular motifs on the surface of microbes (pathogen associated molecular patterns,

(PAMPs)) [49]. In contrast, the acquired immune system needs more than 4 days upon pathogen encounter to develop maximum efficacy [46]. Nevertheless, receptors of the acquired immunity are formed by genetic recombination and recognize specific details of molecular structures, thereby conferring very high specificity and ability to recognize any possible pathogen [46].

3.6 Innate immunity

Cellular constituents of innate immunity are: macrophages, natural killer cells (NK cells), granulocytes (neutrophils, eosinophils, basophils), and dendritic cells (DC) [46].

Macrophages recognize PAMPs on microbes via toll like receptors (TLR) and phagocytize them [50]. The phagocytized pathogens are lysed and the processed peptides of the pathogen (antigens) are presented to the T-lymphocytes on major histocompatibility complex (MHC) molecules [51]. Activated macrophages also produce proinflammatory cytokines: interleukin-1 (IL-1), -6 (IL-6), and tumor necrosis factor alpha (TNF-α). In addition, macrophage derived interleukin-12 (IL-12) is responsible for the development of naive T-lymphocytes to T-effector cell of type 1 (TH1) [52, 53]. The very effective feature of macrophages and neutrophils against pathogens is the inducible nitric oxide synthetase (iNOS) that is responsible for the production of antimicrobial active nitric oxide (NO) [46]. However, higher levels of NO and reactive oxygen species (ROS) produced by macrophages and neutrophils are associated with immunopathologies e.g. tissue injury, inhibition of T-cell proliferation in DP-BB rats, and type 1 autoimmune diseases [54-58].

Neutrophils and eosinophils eliminate invaders either by phagocytosis or by releasing the toxic substances from their granules [59]. Basophils do not phagocytize and release only pharmacological active substances e.g. histamine [59]. Granulocytes are important in the defense against helminthes but have also been associated with immunopathologies e.g. allergy, corneal haze, tropical pulmonary eosinophilia, candidiasis [60].

Granular lymphocytes are natural killer cells (NK). NK are very effective in killing virus infected, altered host cells or antibody marked cells via antibody dependent cell - mediated cytotoxicity (ADCC)) [61, 62]. IFN-γ and TNF-α production by NK cells activates dendritic cells [63].

Dendritic cells (DCs), also known as professional antigen presenting cells (APCs), are the key link between innate and acquired immunity [46]. DCs phagocytize antigens (pathogen), process, and present antigens to naive T-lymphocytes [64]. DCs are the only APCs that can induce primary immune response via antigen presentation and T-lymphocyte activation [64]. In addition DCs activate resting B-lymphocytes by transferring the processed antigen to B-lymphocytes [65, 66].

3.7 Acquired immunity

Acquired immunity consists of T (thymus-derived) and B (bursal or bone marrow–derived) lymphocytes [67]. Both cell types have highly specific receptors that recognize details of molecular structures from a variety of pathogens [46]. This high diversity (5×10^{16} antigen specific receptors) is generated through rearrangement of immunoglobulin gene segments [46, 68, 69]. The diversity is generated in order to recognize a high number of pathogens displaying non-self-structures. However T- and B-clones recognizing self-structures are inactivated in the periphery or eliminated in the thymus and bone marrow, respectively [46, 70]. The inactivation or elimination of self-reactive T- and B-lymphocytes results in the so-called self-tolerance [71]. When self-tolerance is lost, an immune response directed against self-antigens develops resulting in autoimmunity [72]. Factors contributing to autoimmunity include the release of cryptic antigens, genetic predisposition, and infections with pathogens [46].

3.8 B-lymphocytes

B-cells recognize and internalize whole antigens via B-cell receptor (BcR) mediated endocytosis [46]. The internalized antigen is processed and presented to antigen specific T-lymphocytes on class II MHC molecules. The activated T-lymphocyte activate B-lymphocytes [46]. The activated B-lymphocytes proliferate to amplify the antigen specific immune response and secrete low affinity antibodies of class IgM [73]. Upon interaction of antigen specific B- and T-lymphocytes a class switch from IgM to IgG isotype occurs [46, 74]. In addition, B-lymphocytes undergo somatic hypermutation of immunoglobulin genes, thereby generating high affinity antibodies of IgG isotype [75]. This

process is known as affinity maturation and is driven by the mechanisms of somatic hypermutation and selection of high affinity clones [76]. The B-lymphocytes develop either into antibody producing plasma cells or memory cells that respond very fast with the production of antigen specific IgG antibodies upon secondary encounter of the same antigen [46, 77].

Some antigens are able to activate B- lymphocytes in a T-lymphocyte independent manner [78]. In addition to proinflammatory cytokines, TNF-α and IL-6, B-lymphocytes produce also IL-10, in order to suppress the T-lymphocyte immune response driven autoimmunity [79]. Immunopathologies associated with self-reactive B-lymphocytes are e.g. rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis [80, 81].

3.9 T-lymphocytes

Functionally different T-cell subsets, like cytotoxic, helper, and regulatory cells represent the cellular branch of acquired immunity [46].

Cytotoxic T-lymphocytes (CTL) get activated upon interaction with antigen presented on class I MHC molecules of APCs [82]. For the CTL activation the presence of IL-2, which is produced by effector T-lymphocytes, is essential [46]. Primed CTL clonally expand and induce apoptosis of infected or altered (tumor) cells either directly via fas/fasL pathway or indirectly by release of cytotoxins [83, 84]. Increased number of CTLs is associated with the outcome of sepsis and immunopathologies during viral infections [85, 86].

T-helper (Th) cells are involved in T-dependent antibody production by B-lymphocytes. They are also involved in the activation of CTLs as well as innate immune cells [74, 87].

Upon interaction with presented antigen on APCs and co-stimulatory signals, T-helper cells get activated and proliferate [87]. Depending on targeted antigen and cytokine environment, T-helper cells develop into one of the three in human and mice known subsets, namely Th1, Th2 and Th17 [88]. In pigs, only Th1 and Th2 subsets have been described [89]. These subsets orchestrate the acquired immune response to maintain the balance between protective and pathological immune response. Both cell subsets Th1 and Th2 are defined through distinct secreted cytokine profiles [87, 88].

3.10 Th1 and Th2 response

Th1 cell subsets produce IFNγ and IL-2, activate macrophages and ensure survival of memory CTLs [46]. Activated macrophages produce NO, ROS and IL-12, whereas the latter inhibits the development of Th2 response and favors Th1 subset development [53]. An appropriate Th1 immune response is very effective in killing bacteria, protozoa, viruses and fungi. But, an overwhelming response of Th1 type leads to tissue damage and autoimmunity [46, 88].

Th2 cells produce IL-4 and IL-13, activate granulocytes and tissue repairing macrophages. They also induce class switch of IgG production by B-lymphocytes [46]. Th2 mediated immune response is beneficial in clearance of nematodes and infections with persistent antigens [53, 88]. However, the overshooting response of Th2 type is associated with allergy development. The outcome of the disease depends on the Th1 and Th2 effector response generated [46]. Previous studies have shown that the activation of one of the 2 response types is essential to defeat the invader, whereas the other one is rather harmful or even fatal [88]. For example, Th2 immune response elicited by BALB/c mice to *Leishmania major* infection proves fatal. Other mice strains develop Th1 immune response characterized by iNOS induction in macrophages. These mice strains in contrast to BALB/c mice heal from the infection [90-92].

Mycobacterium tuberculosis (*M. tuberculosis*) infection represents an example were the precise balance between Th1 and Th2 response is critical for the outcome of the disease [88]. Th1 immune response with activated macrophages is necessary to keep the intracellular infection under control and to form granulomas [93, 94]. Concurrent Th2 immune response is required to prevent tissue damage from overshooting Th1 immune response [88, 95]. However a premature Th2 immune response to *M. tuberculosis* leads to enhancement of the disease, immunopathology, weight loss, and fibrosis [96]. The Th1-to-Th2 shift was shown to depend on the load of heat-killed (HK) *Mycobacterium bovis* bacillus Calmette-Guerin (BCG). Lower bacterial load induces the protective Th1 immune response. Whereas, a higher doses of HK-BCG induces a shift to Th2 immune response, which is characterized by the production of antibodies to mycobacterial 65-kDa (hsp60) heat shock Protein [97]. Antibodies to HSP65 are described to cross react with host heat shock proteins and to play a role in autoimmune diseases [97].

T-regulatory (Treg) cell subset represents an additional mechanism of peripheral tolerance. They down-regulate the amplitude of an immune response to prevent inflammation and autoimmunity. T-regulatory cells suppress T-helper cells via cell-cell contact (Fas and Granzyme B), production of soluble suppressive factors (IL-10; TGF-β) and competition for growth factors [46, 98].

3.11 Endothelium and pathogens

Endothelium lines the inner layer (*intima* layer) of blood vessels and is composed of endothelial cells (ECs) that are connected via intercellular junctions to enable specific permeability [99, 100]. Endothelial cells are type of epithelial cells and are involved in immune response processes [99]. A study of Wang and co-workers evidenced the existence of embryonic precursor of endothelial and hematopoietic (multipotent blood cell precursors) cells. This report supports the view of the endothelium as an immunological organ [99, 101].

The endothelium is a dynamic organ and a barrier between vascular space and surrounding tissue [99, 102]. The house-keeping functions assigned to endothelium are vasoregulation, maintenance of blood circulation, coagulation, control of nutrients exchange, and inflammatory responses [102, 103].

The epicellular or intracellular interaction of pathogens with ECs induces the secretion of proinflammatory cytokines, chemokines, and the expression of cell adhesion molecules (CAMs), resulting in an inflammatory and procoagulant phenotype of ECs [102, 104].

Several pathogens are able to trigger inflammatory responses and coagulation processes and disrupt the protective function of the endothelium [105]. Pathologies associated with endothelium targeting pathogens are: inflammation of the endothelium, intravascular coagulation and multiple organ dysfunctions, severe sepsis, septic shock, and hemorrhagic diathesis (bleeding tendency) [105].

4 Objectives of the thesis

The hemotrophic *Mycoplasma suis* is the most common cause of acute and chronic infectious anemia in pig (IAP) [33]. Due to the unculturability of *M. suis in vitro* the knowledge about the underlying pathomechanisms is rather basic. *M. suis* establishes persistent infections in pig by evading and modulating the host immune responsiveness [13, 16].

To date, only few pieces of the persistence puzzle are identified e.g. immunomodulatory effects as a result of *M. suis* infection have been shown on both T- and B-lymphocytes. *M. suis* infections have been shown to suppress T-lymphocyte blastogenic responses, indicating an alteration of T-helper lymphocyte activities [36]; Parallel to this, *M. suis* infections also lead to a polyclonal B-lymphocyte activation and the production of autoreactive IgM (cold agglutinins) as well as warm autoreactive IgG antibodies [13].

To fully understand the mechanisms of *M. suis* induced immune modulation, the immunomodulatory effect of *M. suis* on host immune response components needs to be clarified.

In addition, IAP is accompanied with hemorrhagic diathesis ,coagulation dysfunction, petechial bleeding and ecchymosis [106] These clinical signs were attributed to intravasal coagulation and subsequent consumption coagulopathy in one former study [106]. However they cannot fully explain the process responsible for the pathology. Endothelial cells are known to be a key barrier in physiologically intact hosts [105], and the disturbed protective function of endothelial cells almost certainly leads to the development of hemorrhagic diathesis and disseminated coagulation in *M. suis* infections.

The aim of this thesis was to analyze *M. suis*-host interaction in order to get more insights into the mechanisms underlying modulation of host immune responsiveness and induction of hemorrhagic diathesis in *M. suis* infections.

Objective 1. Modulation of the host immune responsiveness by *M. suis* infection

The first research question addressed in this thesis concerned the identification of immune system components that are targeted by *M. suis*. The performed analysis aimed at elucidating the effect of

M. suis on immune response via direct interaction of *M. suis* with immune cells (e.g. invasion and direct damage) or indirect mechanisms (e.g. alteration of the cytokine expression profile).

Objective 2. The role of endothelial cells in development of hemorrhagic diathesis during *M. suis* infection.

This part of the thesis includes the analysis of the interaction of *M. suis* with endothelial cells, the elucidation of EC response to the infection, and the influence of the endothelial activation on coagulopathy.

The study dealing with "Objective 2" is attached as a manuscript: Sokoli A, Groebel K, Hoelzle K, Amselgruber WM, Mateos JM, Schneider MK, Ziegler U, Felder KM, Hoelzle LE. (2012). ***Mycoplasma suis* infection results endothelial cell damage and activation: new insight into the cell tropism and pathogenicity of hemotrophic mycoplasma.** Veterinary Research submitted "under review".

4.1 Objective 1. Modulation of the host immune responsiveness by *M. suis* infection

4.1.1 Materials and Methods

4.1.1.1 Experimental infection

M. suis infection of pigs was performed in accordance with Swiss legislation for animal welfare (Veterinary Office of Zurich, Switzerland; approval 55/2007; 68/2009).

Weaned 4-week-old piglets (N = 4) were housed in the stables of the Institute for Veterinary Bacteriology, University of Zurich, Switzerland. The *M. suis* negative health status was confirmed by a quantitative PCR [32] and ELISA [107]. After two weeks the pigs were splenectomized as described by Heinritzi, 1990 [35] and allowed to recover for additional two weeks. At the age of 8 weeks pigs were infected with *M. suis.* Pig No 6019 was infected intramuscularly with 2 ml of *M. suis* positive blood (10^6 cells/ml) and developed acute course of the disease. In order to obtain the chronic course of the disease the pig No 6043 was infected orally (using *M. suis* contaminated food ≈ 10^{12} cells/ml). For infection, strain 3804 [GenBank: FN984917.1] was used. Daily, the feeding behavior, body temperature, and clinical signs were documented [18]. The clinical status of each pig was assessed using a scoring system described by our group [41]. The score one was given for the occurrence of one of the following clinical signs: fever (> 40 °C), reduced food uptake, lethargy, pale skin, and ear necrosis. Upon occurrence of four clinical signs per day the score four was given and the pig was treated with tetracycline (40 mg/kg body weight) and glucose (35 g/l drinking water), or euthanized. *M. suis*-negative pigs were monitored similarly.

4.1.1.2 *M. suis* antigen preparation

For the *in vitro* experiments, *M. suis* cells were purified from the blood of an experimentally infected pig as described before [43]. Briefly, EDTA anticoagulated blood was centrifuged at low speed (500 x g for 20 min at room temperature (RT)) to separate blood cells from the plasma. The supernatant containing *M. suis* cells was centrifuged at high speed (20` 000 x g) for 1 h at 4 °C

(Hettich Rotixa/AP; Hettich, Germany). The pellet was washed once (same conditions) with ice-cold phosphate buffered saline (PBS, Biochrom). The *M. suis* load was quantified by quantitative PCR analysis (LightCycler™ 2.0 System; Roche Diagnostics, Switzerland; [32]. Blood from an *M. suis*-negative pigs was prepared accordingly to obtain the negative control preparation. *M. suis* antigen and negative control preparation were suspended in PBS and stored in 1 ml aliquots at -80 °C until use.

4.1.1.3 PBMCs

Peripheral blood mononuclear cells (PBMCs) are blood cell with a round nucleus. PBMCs consist of following cell population: The T- and B-lymphocytes, natural killer (NK) cells, monocytes, dendritic cells. PBMCs were extracted from fresh blood of *M. suis* infected pigs and from *M. suis*-negative pigs using Vacutainer cell preparation tubes (BD Biosciences, Switzerland) according to manufacturer's instructions.

The PBMCs from *M. suis*-negative pigs used for the *in vitro* stimulation assays were cryopreserved using 10 % dimethyl sulfoxide (DMSO, Sigma-Aldrich, Switzerland) and stored at -150 °C until use.

The PBMCs used for flow cytometry and light microscopy analysis were fixed in 4 % Formaldehyde (FA, Biochrom, Switzerland) in phosphate-buffered saline (PBS, pH 7.3) for 2 h at RT. FA fixed PBMCs were stored in PBS at 4 °C until further processing.

For scanning electron microscope (SEM) analysis, PBMCs were seeded on cover slips in a 12-well tissue culture plate. PBMCs were incubated in RPMI 1640 medium (Roswell Park Memorial Institute; Biochrom, Switzerland) supplemented with 10 % inactivated fetal calf serum (FCS, Biochrom) and 50 mg/ml ampicillin. After 24 h of incubation at 37 °C the adherent PBMCs were fixed in 2.5 % phosphate-buffered glutaraldehyde (GA, Sigma, Switzerland) for 2 h. GA fixed PBMCs were stored in PBS at 4 °C until further processing.

4.1.1.4 Scanning electron microscopy of PBMCs

The GA fixed PBMCs (adherent on cover slips) were post-fixed in 1 % osmium tetroxide (Fluka Chemie, Switzerland) for 1 h at RT and were then dehydrated in graded-ethanol series. The dehydrated PBMCs were critical point dried (BAL-TEC CPD 030, Critical Point Dryer, Balzers, Liechtenstein) and coated with 12 nm of platinum using the BAL-TEC MED 020 coating system. The samples were mounted on aluminum stubs and analyzed on a Zeiss Supra 50 VP (Zeiss, Germany).

4.1.1.5 Apoptosis and necrosis assays

Naive and activated PBMCs were stimulated with purified *M. suis* antigen preparation (10^4 *M. suis* cells/ml) for 2, 24, and 48 hours. In order to test the immune response of PBMCs to *M.suis* in activated versus naive state 100 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich, Switzerland) was used to induce the activated state of PBMCs [108]. *M. suis* negative blood preparations were used as a negative control. LPS at the concentration of 50 μg/ml was used as a positive control (R Otton 2004 diabetes). For the staining procedure Annexin-V-FLUOS Staining Kit (Roche, Switzerland) was used. With Annexin-V early stages of apoptosis are detected because it binds to phosphatidyserines, which are translocated from the inside to the outside of the plasma membrane in the very early stage of apoptosis. To detect necrosis propidium iodide was used which stains DNA only in permeabilized cells. Cells were analyzed for apoptosis by measuring the amount of bound AnnexinV- FITC or propidium iodide staining of the nucleus with flow cytometry (FACS Canto, BD Bioscience, Switzerland). Single Annexin-V-FITC stained cells were considered apoptotic. Cells stained with propidium iodide or with propidium iodide and Annexin-V-FITC were considered necrotic.

4.1.1.6 Analysis of *in vivo M. suis* – PBMCs interaction

FA-fixed PBMCs from experimentally infected pigs were permeabilized for 20 min using saponin-containing buffer (perm/wash buffer, BD Bioscience, Switzerland). Non-reacted aldehydes were blocked for 10 min with 0.1 M glycine (Carl Roth, Germany) in perm/wash buffer. To reduce the

unspecific binding of antibodies cells were incubated for 1 h in blocking buffer (3 % BSA in perm/wash buffer).

M. suis was specifically stained for 2 h using an anti-MSG1 mouse monoclonal antibody (diluted 1:100 in staining buffer, i.e. 1 % BSA in perm/wash buffer). The anti-MSG1 monoclonal antibody had been produced in our laboratory. The unbound antibodies were removed by washing the cells three times with perm/wash buffer. Cells were then incubated for 1 h with alexa-fluor 647 conjugated goat anti mouse IgG (diluted 1:1000 in staining buffer Sigma-Aldrich, Switzerland). Finally, the PBMCs were washed three times with perm/wash buffer to remove free antibodies.

M. suis positive PBMCs were quantified by flow cytometry (FlowSight, Merck Millipore, Switzerland). To sort the *M. suis* positive PBMCs from *M. suis* negative ones, the fluorescence activated cell sorting aria III (FACS aria III) flow cytometer was used (BD Bioscience, Switzerland). Actin from the sorted *M. suis* positive and negative PBMCs was stained using phalloidin-FITC (1:80 in PBS, Invitrogen, Basel, Switzerland).

Stained cells were centrifuged (cytospin 2, Shandon, Pittsburgh, USA) for 5 min at 1200 g to coverslips that were subsequently mounted on slides using antifade mounting medium (Dabco mounting medium, Sigma-Aldrich, Switzerland).

Samples were analyzed using a confocal laser-scanning microscope Leica SP5 (CLSM, Leica Microsystems). The microscopic images were processed using Imaris 7.5.2 (Bitplane, USA) and Adobe Photoshop CS5.

4.1.1.7 *M. suis* specific proliferation assay

To test the actual proliferation frequency that occurs in naive cells, PBMCs were stained with 5 mM carboxyfluorescein succinimidyl ester (CFSE, Santa Cruz, USA) as described elsewhere [89]. Briefly, 5 - 6 x 10^5 PBMCs per well were incubated with purified *M. suis* antigen (≈ 10^4 *M. suis* cells) for 2, 3, and 5 days. As a positive control LPS at a concentration of 100 ng/ml was used. The blood preparation from *M. suis* negative pig was used as a negative control. The CFSE dilution assay reflects

the proliferation profile of each PBMC subset since the intensity of the detected fluorescence signal is halved with each cell division.

PBMCs of different functionalities are distinguished by the presence of subtype specific receptors on their surface. These receptors are called cluster of differentiation (CD) and used as markers in immunophenotyping [109]. T-helper cells and cytotoxic T-lymphocytes (CTLs) are CD4+ and CD8+, respectively. The B-lymphocytes express CD21 on their surface.

PBMCs were labeled primary with mouse anti – pig CD4, CD8, and CD21 (1:100; Santa Cruz Biotechnologies, USA) and secondary with goat anti mouse IgG conjugated to apc - Cy7 (1:1000) (Santa Cruz Biotechnologies, USA). The obtained data were analyzed with FlowJo 7.6 software (TriStar, USA). The FlowJo software was used to generate the proliferation profile models and to calculate the proliferation index (PI). The PI results from the calculation of the total number of divisions that occurred divided by the number of cells that divided.

4.1.1.8 *In vitro* stimulation of PBMCs and cytokine production

To characterize the type of a given immune response (e.g. T-helper 1 and 2 cell balance) cytokine profiles were indirectly measured. Namely, the cytokine positive cells were quantified and compared to the negative control. The cytokines included in this study are the Th1 and Th2 defining cytokines (IFN-γ; IL-4; IL-10; IL-12), the proinflammatory cytokines (IL-1; IL-6; TNF-α) and the T lymphocytotrophic hormone (IL-2). Th1 cells mainly produce IL-12 and IFN-γ, whereas Th2 cells produce IL-4 and IL-10. To determine the cytokine production by PBMCs, purified *M. suis* antigen preparation was added to thawed naive and activated PBMCs and incubated for 24 h, 3 days and 5 days in RPMI 1640 supplemented with 10 % FCS. The activation state of PMBC was induced either by the combination of Phorbol Myristate Acetate (PMA; 20 mg/ml) and Ionomycin (150 ng/ml) (PMA/Ionomycin, Sigma-Aldrich, Switzerland) or LPS (100 ng/ml). LPS was used as a positive control at the concentration of 100 ng/ml, in order to stimulate the production of the following cytokines: IL-1, IL-6, IL-10, IL-12, TNF-α, IFN-γ. The PMA (20 mg/ml) and Inomycin (150 ng/ml) were used to stimulate the IL-2 and IL-4 production in PBMCs and represent the positive control for

IL-2 and IL-4 assays. As a negative control *M. suis* negative blood preparations were used. During the last 4 h of incubation the metalloprotease blocker brefedin (BD Bioscience, Switzerland) was added, in order to block the cytokine transport by the golgi apparatus. The intracellular cytokine staining was performed using the Cytofix/Cytoperm kit (BD Bioscience, Switzerland) according to the manufacturer's protocol and monoclonal antibodies. The mAbs were diluted 1:100 (Santa Cruz Biotechnologies, USA).

4.1.1.9 Statistical analysis

Statistical Package for the Social Sciences (SPSS 20.0.0) statistical software was used to test differences between groups in the PI values, number of cytokine positive cells, and apoptosis / necrosis rates. Differences were calculated using the unpaired Wilcoxon test and a p value ≤ 0.05 was considered statistically significant.

4.1.2 Results

4.1.2.1 PMBC count during experimental *M. suis*-infection

To analyze the influence of *M. suis* on PMBCs *in vivo,* we determined the changes in PBMCs count during the *M. suis* induced IAP. PBMC counts were measured at different time points, starting two months prior to *M. suis* infection and continuing during the infection until euthanasia.

Pig No 6019 was infected parenterally (intramuscularly) according to the well-established splenectomized *M. suis* pig model [41, 107] and represented the acute course of an *M. suis* infection (IAP). In contrast, pig No 6043 was infected orally (per os) and represented the chronic course of IAP. In both, acutely and chronically diseased pigs the number of PBMCs decreased after the infection with *M. suis* (Fig. 1). In pig No 6019 the PBMC numbers decreased continuously from 1.3×10^4 PBMCs/mm^3 blood (days post infection (DPI) 0) to 7.6×10^3 PBMCs/mm^3 blood (56% of the initial count) on DPI 12. At DPI 12 pig No 6019 had to be euthanatized due to bad conditions (i.e. high fever (42 °C), hypoglycemia, lethargy, reduced food uptake, pale skin) and failure of antibiotic treatment. In contrast pig No 6043 showed the characteristics of a chronic course of the anemia. The PBMC count in pig No 6043 fluctuated: after the infection the PMBC numbers increased from 2.1×10^4 PBMCs/mm^3 blood to 2.5×10^4 PBMCs/mm^3 blood on DPI 17 and then decreased to 1×10^4 PBMCs/mm^3 blood (30% of the initial count) on DPI 28 (end of the experiment).

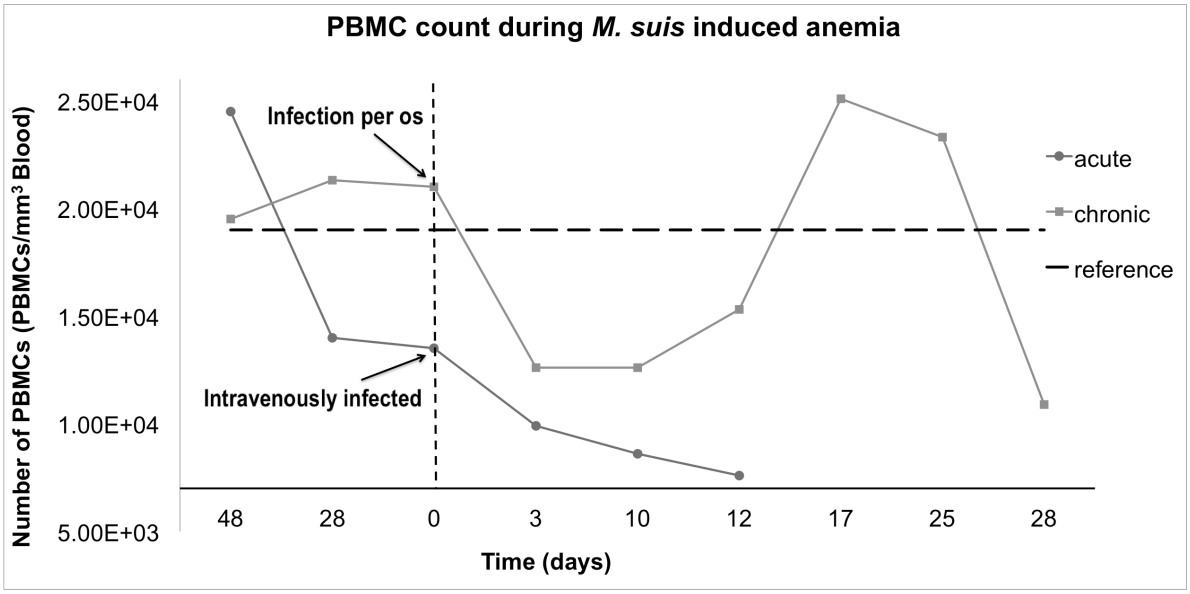

Figure 1: PBMC count during the acute and chronic course of the IAP. The intravenously infected pig No 6019 represents the acute course of IAP (cf. acute). Pig No 6043 was infected per os and represents the chronic course of IAP (cf. chronic). The reference level shows PBMCs count measured under healthy conditions elsewhere [110].

4.1.2.2 Cytopathic effects of *M. suis* on PBMCs *in vitro*

Apoptotic and necrotic effects of *M. suis* on naive and activated PBMCs was tested *in vitro* to determine whether a direct *M. suis* interaction plays a potential role in leukopenia. The apoptosis and necrosis rates were analyzed using annexin-V staining and FACS analysis. In order to test the immune response of PBMCs to *M. suis* in activated versus naive state, 100 ng/ml LPS was used to induce the activation state of PBMCs [108]. Apoptosis and necrosis were determined 2h, 24h, and 48h post *M. suis* stimulation to investigate early, medium, and late events. Early point apoptosis rate was determined after 2h of incubation. At this time point the incubation with *M. suis* increased apoptosis and decreased necrosis in naive porcine PMBCs. In contrast, *M. suis* incubation decreased apoptosis and increased necrosis in LPS activated PMBCs.

At time point 24h (medium time point) the *M. suis* incubation decreased the apoptosis rate and increased the necrosis rate in both, naive and activated PBMCs (Fig. 2 C).

The late cell death rates, after 48h of co-incubation with *M. suis,* were characterized by decreased levels of apoptosis and increased levels of necrosis. The found necrosis rate was less increased in the naive PBMCs when compared to the activated PBMCs.

Differences between *M. suis* incubated and control PMBCs were analyzed in regard to significance using the statistic tests described in the part "Material and Methods".

Overall, the medium and late apoptosis events were significantly decreased in naive PBMCs after *M. suis* incubation (24h and 48h; $N = 6$, $p < 0.05$; Fig. 2 E). In activated PMBCs all (i.e. early, medium and late) apoptosis events were significantly deceased (2h, 24h, 48h; $N = 9$, $p < 0.05$)

Early and late necrosis rates of naive PBMCs upon *M. suis* stimulation were significantly lower (2h and 48h; $N = 6$, $p < 0.05$), whereas the early, medium and late necrosis rates in activated PBMCs were significantly higher after *M. suis* incubation when compared to the negative control (2h, 24h, 48h, $N = 9$, $p < 0.05$).

Both, apoptosis and necrosis rates were used to calculate the overall death rates at all time points investigated (2h, 24h, 48h). As shown in Fig. 2D death rates in naive as well as in activated PBMCs were significantly lower after 24h and 48h when compared to the negative control ($p < 0.05$, $p < 0.05$).

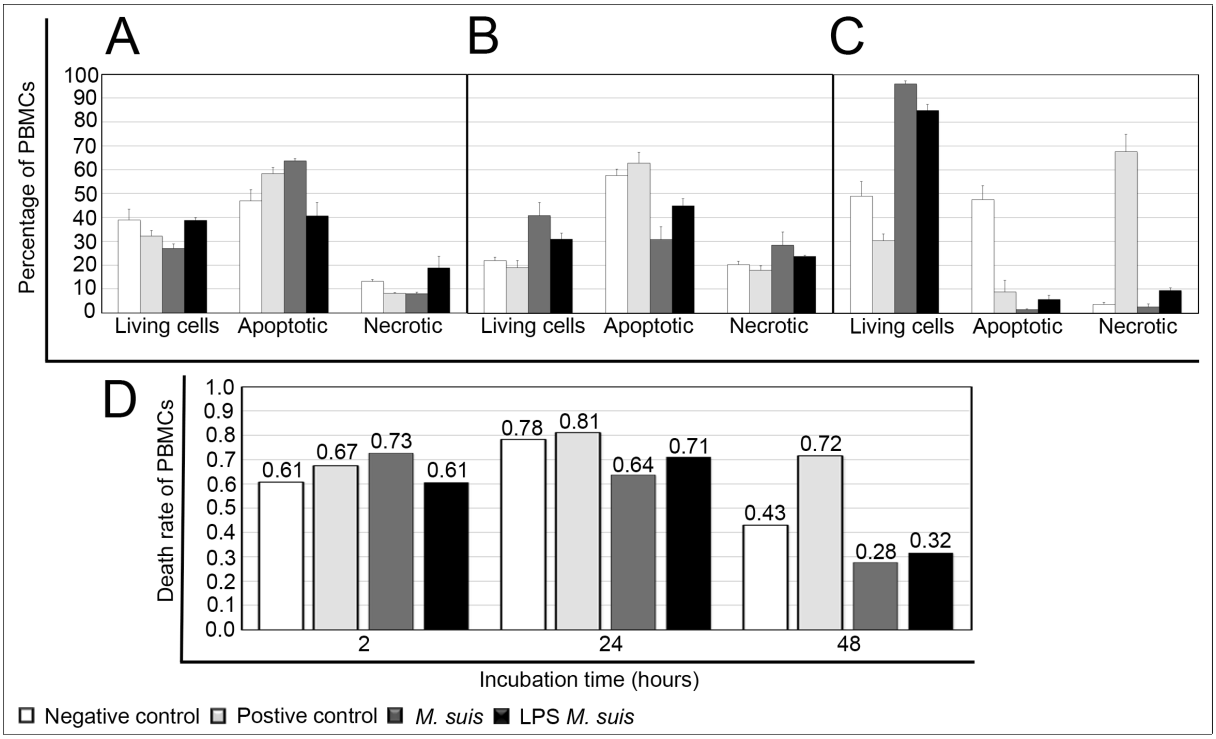

Figure 2: *In vitro* cytopathic effect of *M. suis* antigen on PBMCs. *M. suis* induced apoptosis and necrosis rates of naive and LPS activated PBMCs were analyzed *in vitro* after 2, 24 and 48 h (A; B; C). The death rate was calculated from the fraction of necrotic and apoptotic cells (D). Positive control: PMBCs were incubated with 100 ng/ml LPS. Negative control: PMBCs were incubated with blood preparations from *M. suis* negative pigs. *M. suis* and LPS *M. suis*: Naive and LPS activated PBMCs were incubated with *M. suis.*

4.1.2.3 Analysis of the *M. suis*-PMBCs interaction in infected pigs using scanning electron microscopy

The PBMCs of experimentally *M. suis* infected pig were analyzed by SEM to visualize the effect of *M. suis* infection on PBMCs. The PBMCs were extracted from the acutely diseased pig No 6019 on DPI 12. At this time point crucial clinical signs (i.e. high fever (42°C), hypoglycemia, lethargy, reduced food uptake, pale skin) occurred and the pig had to be euthanatized. At this time point the pig had a bacterial blood load of 3 x 10^9 *M. suis*-cells/mL blood as determined by quantitative LightCycler PCR.

PMBCs were isolated from the blood of the *M. suis* infected pigs according to standard methods as described in “Material and Methods”. The PMBC preparations were characterized by the occurrence

of blood clots (Fig. 2A and B). The majority of the PBMCs were involved in these blood clots, that consisted of erythrocytes, white blood cells, and platelets enclosed in a fibrin network (Fig. 2B). Single *M. suis* cells could not be detected on the surface of PBMCs. Between the PBMCs as well as attached to them, numerous microcolonies of *M. suis* cells of reduced size (100 nm) were observed (Fig. 2C). But underneath the surface of PBMCs many bleb like structures resembling *M. suis* microcolonies were present (Fig. 2D). Some of the PBMCs appeared to be necrotic with released own cell content surrounding them (Fig. 2E). Lesions and blebs of 200 - 800 nm covered their surface (Fig. 2E). Other PBMCs showed cell shrinkage and blebbing that characterizes apoptotic process described elsewhere (Fig. 2F).

No blood clotting, cell debris or cell blebs were observed in the PBMC preparations from *M. suis* negative pigs. The negative PBMCs appeared as single cells (Fig. 2G) or assemblies of 2- 4 cells in close contact, without any fibrin fibers connecting them (Fig. 2H).

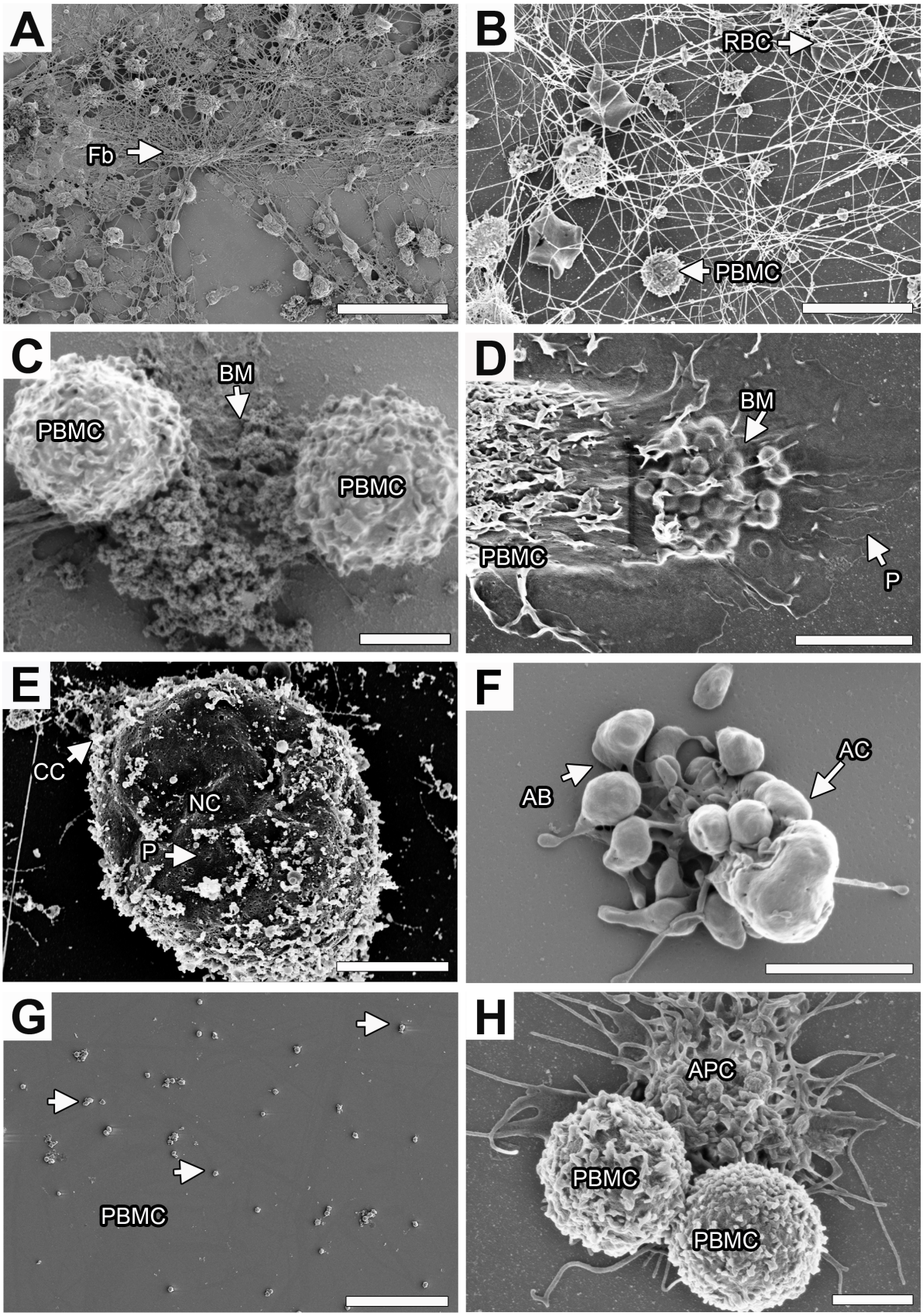

Figure 3: Scanning electron microscopy analysis of PBMCs from *M. suis* infected (A, B, C, D, E, and F) and non-infected (E and F) pigs. A. Overview of PBMCs from *M. suis* infected pig. All cells appear connected

to the fibrin network. Scale bar = 20 μm; B. Higher magnification of a blood clot. RBCs and PBMCs captured in the fibrin network were visible. Scale bar = 10 μm; C. *M. suis* microcolonies of juvenile *M. suis* cells between the PBMCs and attached to them were observed. Scale bar = 2 μm; D. Blebs resembling *M. suis* microcolonies underneath the surface of a PBMCs appeared frequently. Scale bar = 5 μm; E. PBMCs death resembling necrotic patterns was evident and in F. PBMC death characterized by apoptotic morphological changes. Scale bar = 5 μm (E); scale bar = 5 μm (F); G. Overview of PBMCs from *M. suis* negative pig. Single cells as well as cluster of 2 - 4 cells are present in the negative control. No fibrin network was observed in the negative control. Scale bar = 100 μm; H. Higher magnification of PBMCs from *M. suis* negative pig. The cells had the usual morphological characteristics. Scale bar = 2 μm. Abbreviations: AB, apoptotic bodie; AC, apoptotic cell; APC, antigen presenting cell; BM, bacterial microcolony; CC, cell content; Fi, fibrin fibers; NC, necrotic cell; PBMC, peripheral blood mononuclear cells; P, perforation; RBC, red blood cell.

4.1.2.4 Quantification and microscopic analysis of *M. suis* positive PBMCs

M. suis-positive PBMCs from experimentally infected pigs were stained using *M. suis*-specific antibodies [12] and quantified using FlowSight analyzer. PBMCs were extracted from the acutely diseased pig No 6019 on the day of euthanasia (DPI 12). The acute course of the disease was accompanied with a high bacteremia (3 x 10^9 *M. suis*/ml blood). The FlowSight analysis showed that 12 % of the PMBC population was *M. suis*-positive (Appendix I). Since the FlowSight cytometer provides visual verification of the morphology of each cell analyzed and enables gating of cells from debris, the sorted *M. suis* positive PBMCs were whole, intact cells.

Following FlowSight analysis, *M. suis* positive PBMCs were sorted from *M. suis* negative PBMCs by FACS Aria III and stained with FITC-phalloidin (green), in order to visualize F-actin of PBMCs. The sorted cells were than analyzed by confocal laser scanning microscopy.

Similarly to the *M. suis* microcolonies observed by scanning electron microscopy we found *M. suis* microcolonies attached to PBMCs and between PBMCs, clumping - PBMCs together (Fig. 4A and B). PBMCs in contact to *M. suis* microcolonies were covered by *M. suis* single cells (Fig. 4 B and C). Additionally, *M. suis* single cells and cell aggregations were observed inside PBMCs in every stack section throughout the whole volume of PBMCs (Fig. 4C). In addition some of the cells showed

diffuse staining that was spread all over the cell (Fig. 4A). In the negative control no *M. suis* specific signal was observed, neither as a single cell attached or inside PBMCs, nor as a microcolony attached to PBMCs (Fig. 4D and E).

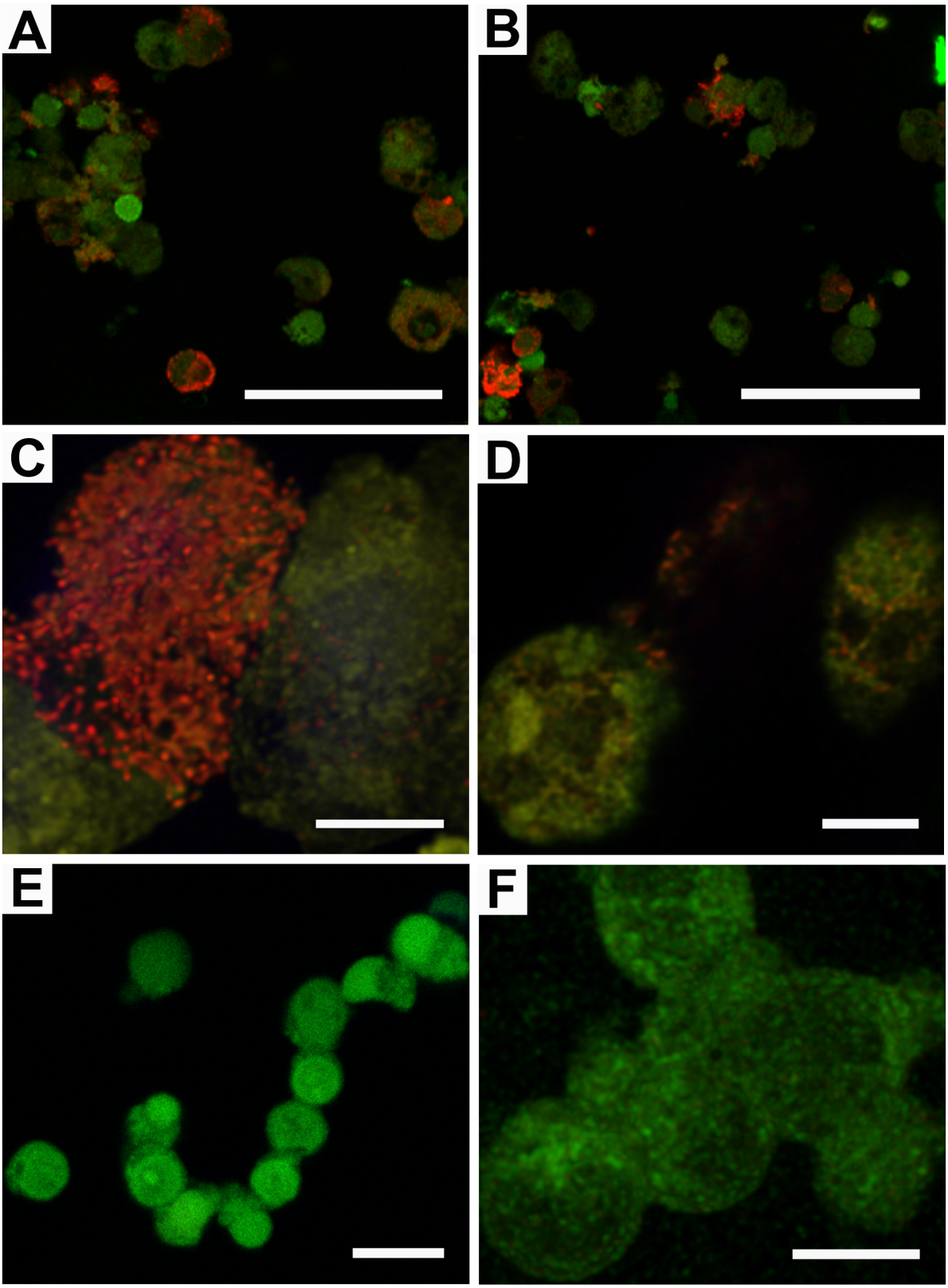

Figure 4: *M. suis* positive PBMCs from an experimentally infected pig. PBMCs were sorted by FACS aria and analyzed by confocal laser scanning microscopy. PBMCs stained for actin with phalloidin-FITC (green) and *M. suis with* anti-MSG1 (red). A. and B. An overview of *M. suis* positive PBMCs. Different staining patterns

were observed e.g. diffuse staining, clearly defined staining located to the plasma membrane, and dotted staining spread all over the PBMC. Scale bar = 50 μm; B. With imaris generated 3D visualization (Imaris 7.5.2) of a *M. suis* microcolony attached to two PBMCs. Scale bar = 5 μm; C. Optical section showing the inside of PBMCs from image B with *M. suis* cells inside. Scale bar = 5 μm; D. Overview of PBMCs from *M. suis* negative pig. No *M. suis* specific signal was detected. Scale bar = 10 μm; D. Higher magnification of PBMCs from an *M. suis* negative pig. Scale bar = 5 μm.

4.1.2.5 *M. suis*-specific proliferation of PMBCs

The *M. suis*-specific proliferation of porcine PMBCs was evaluated using an *in vitro* CFSE dilution assay. Therefore CFSE stained porcine PBMCs were stimulated with purified *M. suis* cells and analyzed after 2, 3, and 5 days respectively (Fig. 6A). Blood preparations from *M. suis* negative pigs were used as negative control. The FlowJo software was used to generate the proliferation profile models and to calculate the proliferation index for total PMBCs, and different PMBC fractions, i.e. B-lymphocytes (CD21+), T-helper cells (CD4+), and cytotoxic T-lymphocytes (CD8+; CTL).

As depicted in Fig. 5 *M. suis* induced a proliferative response of all PBMC fractions, i.e. the total PMBC fraction, T-helper lymphocytes, CTLs, and B-lymphocytes when compared to the undivided CFSE population.

In detail, after 48h *M. suis* stimulated T-helper lymphocytes showed a proliferation index (PI) of 1.2, and therefore, only a slight PI increase of 1.1. The PI of *M. suis* stimulated T-helper cells increased to 1.6 after 3 days and was 1.6 fold higher than the PI of the negative control (Fig. 5 B and C). *M. suis* induced a minor but significant ($p < 0.05$) increase of T-helper cell proliferative response at all time points tested (Fig. 5 B).

The proliferation index of *M. suis* stimulated CTLs (CD8+) increased significantly ($p < 0.01$) and remained elevated with PI $\geq$ 1.4 during the whole incubation period when compared to the negative control (Fig. 5 B and C).

B-lymphocytes (CD21+) showed the highest proliferation index upon stimulation with *M. suis* (Fig. 5B). *M. suis* induced a $\geq$ 2.2 fold increase of the proliferation index of B-lymphocytes ($p < 0.01$).

A negative effect on proliferation response to *M. suis* in B-lymphocytes and CTL`s was observed on the 3rd day of incubation. At the same time point the proliferative response of T-helper cells increased.

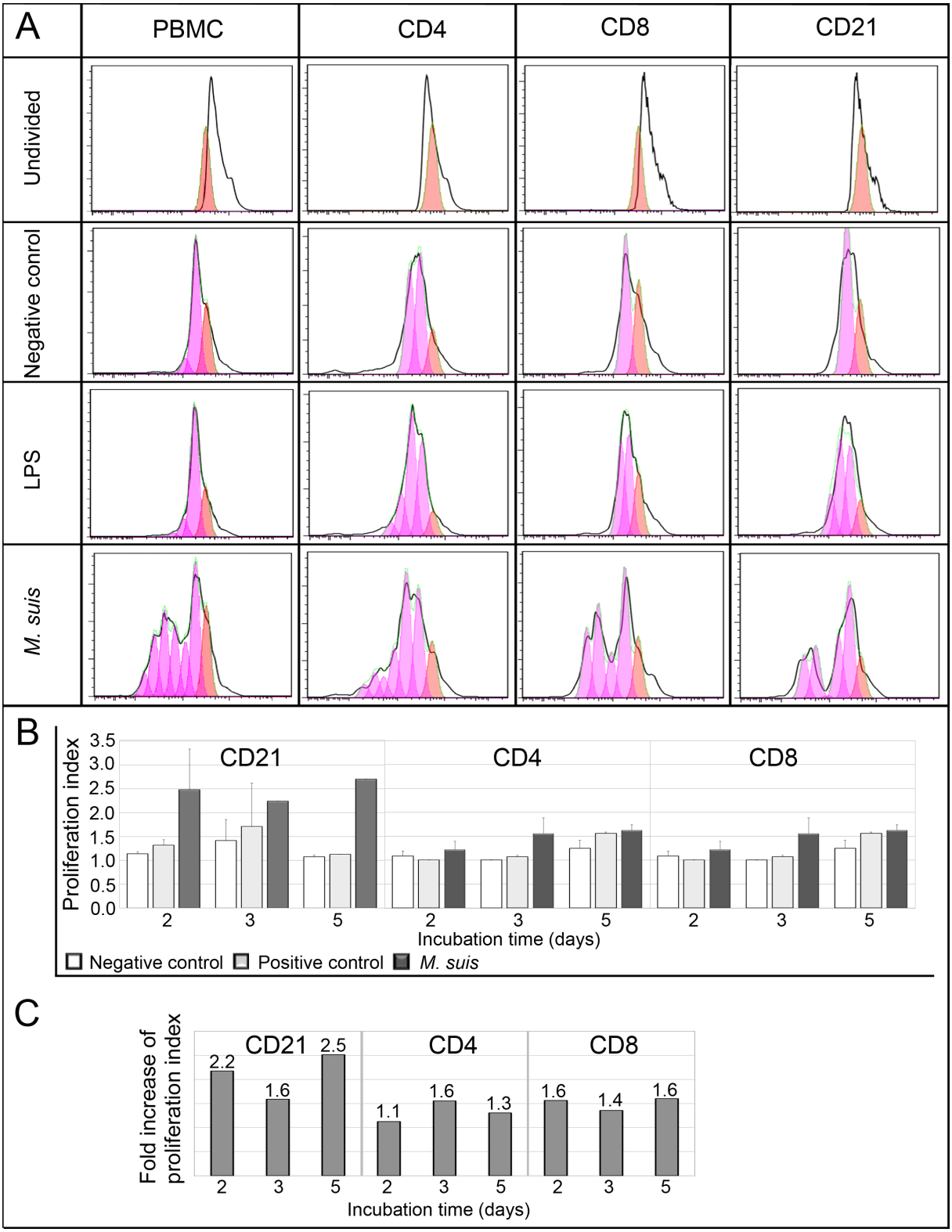

Figure 5: *In vitro* proliferation of PBMCs upon stimulation with *M. suis*. CFSE stained naive PBMCs were stimulated with *M. suis in vitro*. The CFSE profiles were measured on FACS canto II. Proliferation profile models and indexes (PIs) were generated with the FlowJo software. A. Proliferation profile models depicting numbers of resulting daughter populations (peaks) 3 days post stimulation with *M. suis*; Calculated PIs from

2, 3, 5 days post stimulation with *M. suis* for CD21+, CD4+, and CD8+ are presented in B; Calculated fold increase of *M. suis* induced proliferative response compared to the negative control is depicted in C for 2, 3, 5 days post *M. suis* stimulation.

Since T-cell proliferation is dependent on the T-lymphocytotrophic hormone interleukin (IL-2) we quantified the number of IL-2 producing cells upon stimulation with *M. suis* cells *in vitro*. Incubation of PMBCs with *M. suis* for 24h did not increase the number of IL-2 positive T-lymphocytes. After 3 and 5 days the incubation with *M. suis* the number of IL-2 producing cells moderately increased (Fig. 6). The *M. suis* induced up-regulation of IL-2 positive cells remained lower when compared to the PMA/Ionomycin activated PBMCs at all time points. PMA/Ionomycin was significantly ($p < 0.01$) higher than the negative control (Fig. 6). No significant differences between naive and activated PBMCs in IL-2 production upon *M. suis* stimulation could be observed.

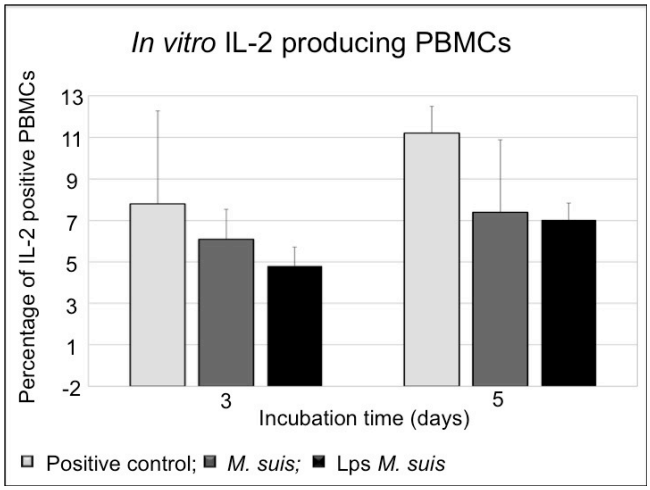

Figure 6: *M. suis* induced IL-2 production in naive and LPS-activated PBMCs. The percentage of IL-2 positive PBMCs upon stimulation with PMA/Ionomycin (positive control) and *M. suis* is shown. The intracellular IL-2 was quantified with FACS canto II.

4.1.2.6 Altered cytokine environment and Th1/Th2 immune response

Together with other factors e.g. strength of T-lymphocytes stimulation and pathogen associated molecular patterns (PAMPs), the cytokine environment determines the T-helper subset development. To get insights into the development of T-helper cell (Th1 and Th2), we stimulated naive and LPS activated PBMCs with *M. suis in vitro*. The number of cells positive for intracellular Th1 and Th2 promoting cytokines was quantified by flow cytometry. Cytokines favoring Th1 immune response are

IFN-γ and IL-12. Th2 immune response beneficial cytokines are IL-4 and IL-10. T-regulatory cells also produce the latter to suppress the immune response of other immune cells.

After 1 day of incubation *M. suis* induced an increase of naive PBMCs positive for all four cytokines tested. Fold increase of Th1 and Th2 cytokine positive PBMCs was ≥ 1.2 higher compared to the negative control (Fig. 7A and B). When PBMCs were activated with LPS or PMA/Ionomycin, the number of cytokine positive PBMCs upon stimulation with *M. suis* remained lower than the number of naive PBMCs stimulated with *M. suis* (Fig. 7A and B).

After 3 days of incubation, IL-10 positive PBMCs dominated: naive PMBCs as well as activated PMBCs reached compared to negative control a 4 and 2.9 (Fig. 7A) fold increase of IL-10 expression, respectively (Fig. 7A, 7B).

From time point 3 days to time point 5 days, the expression of Th1 and Th2 favoring cytokines IFN-γ, IL-4 and IL-10 decreased in naive PMBCs. Nevertheless, the number of IFN-γ positive cells was higher in comparison to the number of IL-4 producing cells, even on the 5th day.

Similar IL-4 and IL-10 expression patterns were observed upon stimulation with *M. suis* antigen in LPS activated PBMCs. However, IL-4 and IL-10 expression was lower in the activated PMBCs compared to the naive cells. Furthermore, IFN-γ expression decreased and IL-12 expression increased in LPS activated PBMCs.

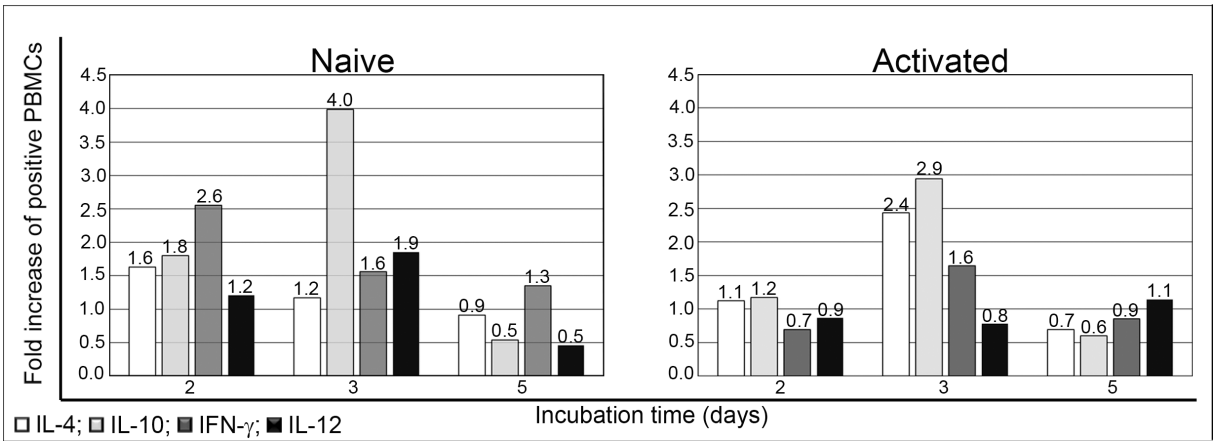

Figure 7: Th1 and Th2 cytokine profiles. Values show the calculated fold increase of cytokine production upon stimulation with *M. suis* compared to negative blood preparation. A. Cytokine profiles induced by *M. suis* antigen in naive PBMCs. B. Cytokine profiles induced by *M. suis* in activated PBMCs. PBMCs tested for IL-4

production were activated with PMA/Ionomycin. The activation of PBMCs tested for IL-10, IL-12, and IFN-γ was induced with LPS.

4.1.2.7 *In vitro* upregulation of proinflammatory cytokines

To test the influence of *M. suis* on proinflammatory cytokine production naive and LPS activated PBMCs were cultivated with *M. suis* antigen. After 1, 3 and 5 days the number of TNF-α, IL-6 and IL-1 positive cells was determined by flow cytometry. The results are illustrated in Fig. 8.

Compared to the negative control *M. suis* induced a significant upregulation of TNF-α, IL-6 and IL-1 ($p < 0.05$; $p < 0.05$; $p < 0.05$). IL-1upregulation upon *M. suis* stimulation could be observed from the first day of cultivation, whereas IL-6 and TNF-α positive cells increased after 3 days of incubation. After 3 days of incubation, *M. suis* induced upregulation of TNF-α positive PBMCs (naive and activated) was even higher than LPS (positive control) induced upregulation. After 5 days the number of TNF-α, IL-6 and IL-1 positive cells were higher in the activated cell fraction when compared to the naive cell fraction.

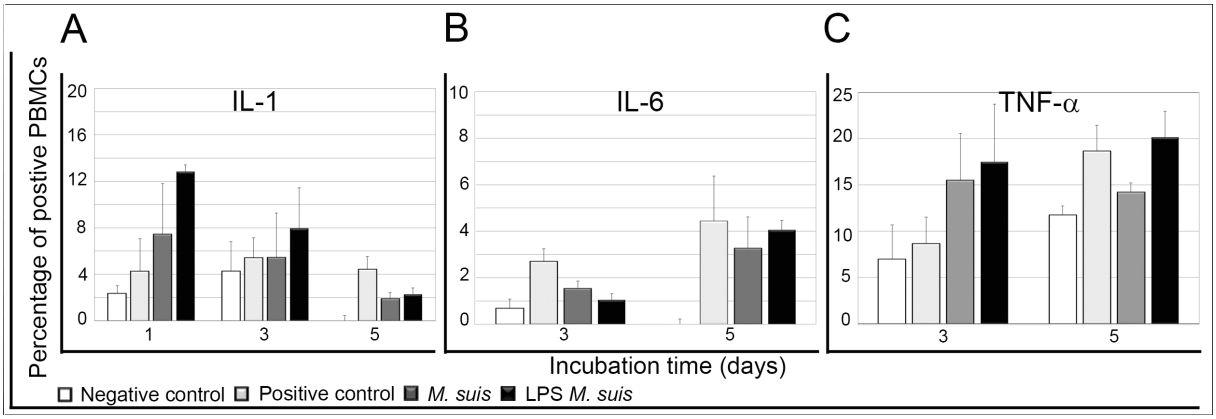

Figure 8: Percentage of proinflammatory cytokine positive PBMCs. *M. suis* induced increase in IL-1, IL-6, and TNF-α positive PBMC number compared to negative control. A. IL-1 production in naive and activated PBMCs upon stimulation with *M. suis. M. suis* induced increase in number of IL-1 positive naive and activated PBMCs. The number of activated PBMCs positive for IL-1 was higher than the number of naive PBMCs positive for IL-1. B. IL-6 production in naive and activated PBMCs upon stimulation with *M. suis.* The number of naive PBMCs positive for IL-6 was higher than the number of activated PBMCs in the first 3 days of

incubation. The opposite was observed after 5 days of incubation. C. *M. suis* induced production of TNF-α in naive and activated PBMCs. *M. suis* induced increase in number of TNF-α positive naive and activated PBMCs. The number of TNF-α positive activated PBMCs was higher than the number of naive PBMCs positive for TNF-α.

4.1.3 Discussion

M. suis has the ability to establish a lifelong persistent infection [13]. However, there are only limited studies to investigate the underlying persistence mechanisms. One former study provided strong evidence to suggest that *M. suis* infections suppress the T-lymphocyte blastogenic responses by inducing an alteration of T-lymphocyte activities. Additionally it was reported that *M. suis* induces a polyclonal B lymphocyte activation and the production of autoreactive antibodies of the IgM and IgG type [36, 39]. This misdirected immune regulation (i.e. immune modulation) helps *M. suis* to avoid elimination and to establish persistent infections in an immunologically hostile environment. However, analysis of immunomodulatory activities of *M. suis* in experimental animals is difficult due to the extraordinary complexity of the system. For this reason detailed studies focusing on *M. suis* effects on single components of immune system are missing so far. The present study was undertaken to analyze the interaction of *M. suis* with PBMCs, to identify immune components targeted by *M. suis* in order to clarify mechanisms that are responsible for the *M. suis* induced modulation of host immune responsiveness.

To obtain first insights we determined the PBMC counts during experimental infection. The acutely diseased pig presented a mild leukopenia whereas the chronically diseased only showed a slight decrease of PBMC count [111, 112]. Both, leukopenia and leukocytosis were reported in *M. suis* infected animals [34, 35, 113]. Interestingly, leukopenia was described as a symptom in human hemotrophic mycoplasma infections, i.e. in one case of human “eperythrozoonosis” [114] and in an infection with the new hemotrophic bacterial species Candidatus M. haemohominis [24]. Leukopenia is a common response to several infections with viruses but also bacteria like *Mycoplasma fermentans* that cause immune modulation of host [115-117]. *M. fermentans* - derived membrane lipoprotein LAMPf was shown to induce leukopenia *in vivo* [115] and cell death of thymocytes *in vitro* [118].

The decrease in PBMCs count upon infection with *M. suis* could be due to an direct cytopathic effect of *M. suis* components on PBMCs or an indirect effect via immunopathology induced by *M. suis* infection. Direct cytopathic effects could involve apoptotic or necrotic cell death. Previous studies have shown that the endonuclease activity of various mycoplasmas can result in an increased sensitivity of PBMCs to apoptosis induction [119]. In our study, we found evidence for a direct

cytopathic effect of *M. suis* on PBMCs via apoptosis and necrosis only in the first 2 h of incubation. At this time point the *M. suis* induced PBMC death rate (consisting of apoptosis and necrosis rate) was distinctly higher than the negative control. However, longer incubation of PBMCs with *M. suis* obviously had an adverse effect on the PBMC death rate: no cytopathic effect of *M. suis* on PBMCs was observed, the total cell death rate rather decreased than increased. Especially the number of apoptotic events was decreased when compared to the controls. Therefore, it could be assumed that *M. suis* may contribute to survival of PBMCs *in vitro* and may act as a kind of prosurvival factor by alleviating proapoptotic signals as it was described for other mycoplasmas [119]. Further studies are necessary to analyze whether the decreased cell death rate an effect of the specific situation in the hemotrophic *M. suis* research is, i.e. *M. suis* do not proliferate *in vitro* and, therefore, increasing bacterial loads or the viability of *M. suis* in infected animals might account *in vivo* for a possible cytopathic effect on PBMCs, that we observed in experimentally infected pigs. This correlation between viability/pathogen replication and the induction of leukopenia was reported for e.g. infections with the human cytomegalovirus infections. Our SEM analyses supported this hypothesis, since PBMCs from *M. suis* infected pig displayed morphological patterns which were highly indicative for necrotic and apoptotic processes, i.e. the release of own cell content surrounding the cells, surface localized lesions and blebs of 200 - 800 µm as well as cell shrinkage and blebbing [119, 120]. Further studies are required to analyze whether *M. suis* has a cytopathic effect on PBMCs. In particular, *in vivo* analysis of the apoptotic and necrotic *M. suis* effects on PBMCs in correlation to the bacterial load profiles would provide further insights. Furthermore, *in vitro* experiments are needed to assess the potential relationship between *M. suis* and PBMC apoptosis or to determine putative specific host cell signaling pathways, which are modulated by *M. suis*.

Interestingly, we observed that majority of PBMCs from *M. suis* infected pigs was embedded in blood clot. The found blood clot formations could also contribute to the observed leukopenia. The clotted PBMCs were interconnected via fibrin network and also connected with *M. suis* infected erythrocytes. These clots were similar to blood clots observed sealing the injuries of the endothelium induced by *M. suis* infection [121]. PBMCs caught by fibrin networks of blood clots were not available as single cells for hematologic measurement therefore the coagulopathy might explain the decreased number of

PBMCs in *M. suis* infection. A previous study has shown that thrombocytopenia induced by *M. suis* infection was attributed to intravascular coagulation and subsequent consumption coagulopathy [106]. Moreover, the endothelium could be involved in the development of leukopenia during an *M. suis* infection: recently we have demonstrated that *M. suis* infections induce activation and destruction of the endothelium [121]. From other studies it is well known that an activated endothelium recruits as well as translocates PBMCs from blood vessels into the inflamed tissue [122]. The possible role of the endothelium in the development of leukopenia during an *M. suis* infection remains to be clarified by e.g. histological analysis of tissue from *M. suis* infected pigs.

M. suis is capable of attaching and invading erythrocytes and endothelial cells [18, 121]. In this study, we wanted to determine whether PBMCs are also a target for attachment and invasion. For other pathogens invasion of PBMCs is well known to contribute to the misdirected immune response and to the persistence of infections [123]. Firstly, we demonstrated by SEM that *M. suis* cells were attached to PBMCs from infected pigs. However, in contrast to infected erythrocytes, we could not find single and regular shaped *M. suis* cells but rather *M. suis* microcolonies attached to PBMCs. These microcolonies consisted of irregular *M. suis* cells of reduced size resembling juvenile and nanotransformed cells described on infected erythrocytes and in *M. suis* cultures, respectively [36, 45, 124]. Secondly, frequently observed blebs underneath the surface of PBMCs resembled intracellular localized *M. suis* microcolonies. Probably the PBMC surface with its numerous surface extensions made the recognition and visual distinction of single *M. suis* cells difficult, especially in case of juvenile forms of *M. suis*. Immunofluorescence labeling and microscopic analysis confirm these findings. *M. suis* microcolonies were found in close contact to PBMCs. CSLM optical sections clearly demonstrated that these PBMCs with attached *M. suis* microcolonies contained intracellular *M. suis* single cells and cell clusters. Attachment to and invasion of PBMCs has also been reported in *M. bovis* [119]. Importantly, these *M. bovis* cells attaching and invading the PBMCs also appeared as clumps, which might resemble a kind of microcolony.

The non-fractioned PBMC population analyzed consists of different cell types e.g. phagocytic host cells (i.e. monocytes and dendritic cells), therefore the presence of both Mycoplasma species (*M. suis* and *M. bovis*) inside the PBMCs might result from an active bacterial process or internalization via

phagocytosis. Therefore, further analyses are required to confirm an invasion process or a phagocytic fusion of *M. suis* with PBMCs. Immunogold electron microscopic analysis will give more insights into the possible mechanisms responsible for invasion or fusion of *M. suis* with PBMCs [125]. Furthermore, our results raise the important question in regards to the involvement of the *M. suis* adhesion proteins MSG1 and α-enolase in the attachment to and invasion in PBMCs [12, 45]. For *M. bovis* the proteins responsible for the attachment and invasion in PBMCs were not identified so far, and for *M. suis*, PBMC adhesion proteins remain to be determined.

M. suis infections resulted in T-lymphocyte suppression and misdirected immune responses [36] supporting the establishment of persistent infections. Our *in vitro* experiments revealed an alteration of blastogenic response of B- and T-lymphocytes in the early phase of *M. suis*-PBMC interaction. *M. suis* antigens induced a high proliferation of naive B-lymphocytes after 2 days and at the same time no significant up-regulation of T-helper cells indicating a T-helper cell independent B-lymphocyte polyclonal activation. This is consistent with previous finding i.e. that *M. suis* induced abrupt decrease of specific immune response was associated with an abrupt increase of autoreactive IgG antibodies [43]. Both facts support the hypothesis that *M. suis* must have a B-lymphocyte mitogen that is able to induce the polyclonal activation and proliferation of naive B-lymphocyte without previous sensitization of lymphocytes [126].

In parallel, the T-lymphocyte proliferative response to *M. suis* was only moderately increased. This result is in line with the report concerning altered T-lymphocyte blastogenic response [36]. Furthermore we found strong evidence for a T-regulatory cell activation and the suppressive effect of T-regulatory cells on T-helper cells, i.e. (1) the observed proliferation profiles of CD4-, CD8-, and CD21-positive cells were typically characterized by an inversely proportional course of the proliferation level of CD4-positive cells to the proliferation levels of CD21- and CD8-positive cells. This inverse proliferation relationship of CD4- positive cells with CD21-, and CD8-positive cells is highly indicative for T-regulatory cell activation and an increased suppressive effect of T-regulatory cells (CD4+ CD25+ foxp3+) on T-helper cells. (2) IL-2 and IL-10 expression patterns were also highly indicative for a T-regulatory cell activation and an increased suppressive effect of T regulatory cells. Immune suppressive and immunomodulative T-regulatory cells suppress T-helper cells and are

important factors during immune responses particularly with regard to autoimmune processes, which are main pathogenic features during *M. suis* infections. T-helper cell suppression by T-regulatory cells via IL-2 consumption, induction of apoptosis and IL-10 production has an important role in preventing autoimmunity and immunopathology [127, 128]. The T-regulatory cell activation is characterized by increased IL-2, and IL-10 levels and by the suppressive effect on other PBMCs [46, 89, 127, 128]. In *M. suis*, the number of IL-2 positive T-lymphocytes increased after 3 days, and most probably as a consequence thereof the proliferative response of naive T-regulatory cells (CD4+) also increased. However the increase was only minor [1]. The *M. suis* induced IL-10 positive T-regulatory cells also reached the highest peak on day 3 and henceforth, the proliferative response of CD4+ (most probably of T-helper type) decreased on day 5. Further analysis are required to confirm the suppressive effect of T-regulatory cells on T-helper cells upon stimulation with *M. suis* and to determine the role of T-regulatory cells during autoimmune processes in *M. suis* infected pigs [89].

Analysis of the cytokine patterns gave valuable information about the Th1/Th2 balance during an *M. suis* infection. Interestingly, the number of IL-10 positive cells decreased on day 5 and no increase of IL-4 positive cells was observed, which indicates that the anticipated Th1/Th2 shift does not occur. Both types of immune response Th1 as well as Th2 seemed to be active in the early phase of the *in vitro* interaction. This result is in accordance to the outcome of a previous immunization study by Hoelzle and co-workers [41]; the immunization of pigs with recombinant MSG1 resulted in production of IgG1 and IgG2. However, the immunization study of Hoelzle and co-workers also documented a Th2 dominated immune response if the pigs were vaccinated with *Escherichia coli* transformants expressing MSG1. And this Th2 dominance was obviously correlated to an increased protection efficacy (i.e. fatality of IAP). According to elevated number of IFN-γ-positive cells compared to IL-4-positive cells, this study suggested a predominance of the Th1 response in the early phase of disease. Therefore, *M. suis* antigens other than MSG1 must be responsible for the induction of Th1 type of the immune response. In addition, an overshooting Th1 immune response leads to immunopathological features observed in *M. suis* infections e.g. disseminated tissue damage and autoimmunity [13, 88, 121]. However, IL-4 and IFN-γ-positive cell number decreased on day 5 of the *in vitro* stimulation with *M. suis*. It is well known, that the acquired immune response needs at least 4

days to develop and is characterized by either Th1 (IFN-γ, IL-12) or Th2 (IL-4) positive cell proliferation [78]. Therefore the *in vitro* induced drop in the IL-4, IL-12, and IFN-γ positive cells after 5 days might represent a possible suppression mechanism of cell mediated specific immune response in *M. suis* infection. Follow up studies are needed to confirm this outcome *in vivo*.

A detailed look at the cytokine expression levels during the interaction of *M. suis* with PBMC *in vitro* provided further insights into some pathogenic features during *M. suis* infections. The decrease of IL-12 positive cells upon *M. suis* stimulation represents a potential mechanism of immune response impairment, since IL-12 is essential for initial bacterial control in infections and IL-12R deficient humans were shown to be highly susceptible to intracellular pathogens [129, 130]. IL-12 is produced by antigen presenting cells (APCs) and has been shown that, in contrast to mouse model, in humans IL-10 affects APCs instead of Th1 cells [131]. Interestingly, pigs have the same mechanism as in humans, the *in vitro* increase of IL-10 positive cells lead to decrease of IL-12 positive APCs, thereby affecting the Th1 immune response indirectly.

M. suis antigen induced an increase of the following proinflammatory cytokines: TNF-α, IL-1, and IL-6. These proinflammatory cytokines enhance the T-lymphocytes response [132]. Increased levels of these multifunctional proinflammatory cytokines were associated with the immunopathology in different infectious disease e.g. dengue fever, meningococcal septic shock and different mycoplasma induced diseases [133-136]. In *M. suis* infected pigs the acute disease is characterized amongst other clinical signs by fever, immunopathology and hemorrhagic diathesis. The high load of *M. suis* in the blood most probably leads to *in vivo* overproduction of proinflammatory cytokines with amplification of inflammatory response of Th1 type. The amplified immune response with enhanced proinflammatory environment leads to activation of APCs and bystander activation of self-reactive lymphocytes like the previously reported production of IgG-autoantibodies to host actin [137]. The proinflammatory environment, especially TNF-α and IL-1, increase leads to observed endothelial activation, widespread damage, and vascular occlusion in *M. suis* infected pigs [106, 121].

This study provides new puzzle pieces that shed light on the *M. suis* persistence strategy and the *M. suis* induced pathology.

The found coagulopathy implicates leukopenia, which represents immune system impairment and supports the persistence of the agent. Even in the early phase of interaction *M. suis* induces T-helper cell inhibition, thereby preventing the generation of *M suis* specific cellular immune responses. The inhibition of T-helper cell activation occurs most probably due to *M. suis* induced increase of IL-10, indicating the activation of T-regulatory cells. During exposure to *M. suis* a rise in the level of IL-10 was accompanied by parallel decline in the production of IL-12, indicating an attenuated phagocytic function of macrophages.

The T-helper cell independent B-lymphocyte polyclonal activation by *M. suis* leads to the production of autoreactive antibodies, resulting in the autoimmunity.

The elevated levels of proinflammatory cytokines upon *M. suis* stimulation lead to the increased activation of innate immunity components and dysfunctional activation of the endothelium. Both conditions contribute to the systemic inflammation and immunopathology. Furthermore, the study provides first evidence that *M. suis* was found attached not only to erythrocytes and free in plasma but also in close association with PBMCs as microcolonies, which could potentially aid transport of *M. suis* to other tissues within the host. In summary, clarification of immune mechanisms as well as host cell tropism and persistence mechanisms will help us in future to develop new strategies for the control and prevention of this economically important disease.

4.2 Objective 2. The role of endothelial cell in hemorrhagic diathesis induced by *M. suis* infection

4.2.1 Manuscript: "*Mycoplasma suis* infection results endothelial cell damage and activation: new insight into the cell tropism and pathogenicity of hemotrophic mycoplasma".

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Manuscript submitted to Veterinary Research

Own contribution:

I performed *in vitro* assays, fluorescence staining procedures and flow cytometry analysis, collected samples from *in vivo* experiments, performed the immunogold staining and examined samples using scanning- and transmission electron microscopy, and wrote the manuscript.

Abstract

Hemotrophic mycoplasmas (HM) are highly specialized red blood cell parasites that cause infectious anemia in a variety of mammals, including humans. To date, no *in vitro* cultivation systems for HM have been available, resulting in relatively little information about the pathogenesis of HM infection. In pigs, *Mycoplasma suis*-induced infectious anemia is associated with hemorrhagic diathesis, and coagulation dysfunction. However, intravasal coagulation and subsequent consumption coagulopathy can only partly explain the sequence of events leading to hemorrhagic diathesis manifesting as cyanosis, petechial bleeding, and ecchymosis, and to disseminated coagulation. The involvement of endothelial activation and damage in *M. suis*-associated pathogenesis was investigated using light and electron microscopy, immunohistochemistry, and cell sorting. *M. suis* interacted directly with endothelial cells *in vitro* and *in vivo*. Endothelial activation, widespread endothelial damage, and adherence of red blood cells to the endothelium were evident in *M. suis*-infected pigs. These alterations of the endothelium were accompanied by hemorrhage, intravascular coagulation, vascular occlusion, and massive morphological changes within the parenchyma. *M. suis* biofilm-like microcolonies formed on the surface of endothelial cells, and may represent a putative persistence mechanism of *M. suis*. *In vitro* analysis demonstrated that *M. suis* interacted with the endothelial cytoskeletal protein actin, and induced actin condensation and activation of endothelial cells, as determined by the up-regulation of ICAM, PECAM, E-selectin, and P-selectin. These findings demonstrate an additional cell tropism of HM for endothelial cells and suggest that *M. suis* interferes with the protective function of the endothelium, resulting in hemorrhagic diathesis.

Keywords: haemotrophic mycoplasmas, anemia, hemorrhagic diathesis, endothelial damage, biofilm formation

Introduction

Hemotrophic mycoplasma (HM) is a global etiological agent of infectious anemia in a variety of animals. HM is a small erythrocytic parasite that adheres to and invades red blood cells (RBCs), which are the exclusive host cell of HM [1-3]. To date, no *in vitro* cultivation system for HM has been established. In the past few years, reports of zoonotic human HM infections have increased markedly. Strains of *Mycoplasma suis*, *M. haemofelis* and *M. ovis* have been isolated from humans [4-6]. Additionally a novel HM species, termed *Candidatus M. haemohominis,* was recently reported in a human subject and was associated with clinical symptoms of pyrexia and hemolytic anemia [7].

In pigs, acute *M. suis* infection (infectious anemia in pigs, or IAP) manifests as hemolytic anemia and hemorrhagic diathesis accompanied by immune modulation and coagulation dysfunction due to intravasal coagulation and subsequent consumption coagulopathy [8-13]. However, disseminated intravascular coagulation cannot fully account for the clinical signs of acute *M. suis* infection, e.g. cyanosis, petechial bleeding, and ecchymosis. One possibility is that the endothelial cells (ECs) of the vasculature are involved in the pathogenesis of IAP. Electron microscopic studies of RBCs from experimentally-infected pigs revealed massive morphological changes as well as strong agglutination of parasitized and non-parasitized cells. Blood clotting in these animals was attributed to the formation of cold and warm autoreactive IgM and IgG antibodies directed against the RBC surface and to an acute phase response of the immune system [8, 13, 14].

To date, several pathogenic bacteria, including *M. mycoides* subsp. *mycoides*, *M. gallisepticum, M. neurolyticum, Bartonella bacilliformis, Rickettsia conorii* and *Bacillus anthracis*, have been described that target and damage the endothelial barrier, or interact closely with EC, triggering inflammatory responses and coagulation processes. In this manner, they are able to interfere with the protective function of ECs through a variety of mechanisms, including invasion and toxin induced endothelial damage, that latter of which could induce hemorrhagic diathesis [15-20].

Based on current evidence, RBCs serve as the exclusive target and host cell of *M. suis*. The goal of the current study was to elucidate potential interactions between *M. suis* and ECs both *in vivo* and *in vitro*. Our hypothesis is that the interaction of *M. suis* with ECs, via either adhesion and/or activation, leads to an activated EC phenotype, thereby mediating damage to the vascular endothelium and activation of

pro-inflammatory and inflammatory cascades. These direct and indirect interactions induce a systemic immune response resulting in endothelial pathophysiology. We demonstrated that *M. suis* closely interacts with ECs *in vivo* and *in vitro*, resulting in endothelial activation and destruction. *M. suis* infection led to endothelial damage, the induction of pro-inflammatory and inflammatory cascades, and immunopathology.

Materials and Methods

Experimental infections

Experimental *M. suis* infection was performed as described previously [21-23] in accordance with Swiss legislation for animal welfare (Veterinary Office of Zurich, Switzerland; approval 55/2007; 68/2009). Briefly, *M. suis*-negative piglets (n = 10) underwent splenectomy and then six of them were infected with an intramuscular inoculation of 2 ml of *M. suis*-containing blood (strain 3806; 1×10^8 *M. suis*/ml blood [1]. Pigs were scored daily for the following parameters: feeding behavior, body temperature, and clinical signs, as previously described [24]. Briefly, a score of 1 was given for each occurrence of reduced food uptake, fever (> 40°C), lethargy, and pale skin/ear necrosis. Individual scores were then summed to arrive at an overall score for each animal. When a score of 4 was reached (clinical attack), pigs were treated with tetracycline (40 mg/kg body weight) and glucose (35 g/L drinking water), or euthanized. *M. suis*-negative pigs were monitored similarly.

Cell culture

Primary porcine aortic endothelial cells (PAEC; European Collection of Cell Cultures, Salisbury, UK) were used for the *in vitro* adhesion assays. The immortalized porcine aortic endothelial cell line PEDSV.15 was used for *in vitro* activation [25]. PAECs were cultured in Porcine Endothelial Growth Medium (CellMade, Archamps, France) containing penicillin (100 U/mL) and streptomycin (100 mg/mL). PAECs between passages three and six were seeded on cover slips in a 24-well tissue culture plate.

PEDSV.15 were maintained in DMEM supplemented with 10 % FCS, 1 mM sodium pyruvate, 1 mM L-glutamine, non-essential amino acids, and 20 mM HEPES (all from Biochrom, Berlin,

Germany) [26]. PEDSV.15 cell culture assays were performed at 37 °C and 5 % CO_2 in 1.5 ml of DMEM in 12-well tissue culture plates.

Purification of *M. suis*

M. suis was purified from the plasma of infected pigs as described previously, with slight modifications [22, 23]. Briefly, sodium citrate anti-coagulated blood was subjected to centrifugation for 5 min at 300 × *g* to sediment the erythrocytes. Plasma was removed and subjected to centrifugation at 20000 × *g* for 1 h at room temperature (RT) (Hettich Rotixa/AP; Hettich, Tuttlingen, Germany). The resulting pellet was washed twice and then resuspended in phosphate buffered saline (PBS, Biochrom). *M. suis* was quantified by LightCycler (LC) PCR analysis [27]. As a negative control, blood from non-infected pigs was prepared using the same procedure (negative control preparation).

Harvesting of blood vessels and parenchyma (heart and liver

Blood vessels (abdominal aorta and veins) and parenchyma (heart and liver) were collected from euthanized *M. suis*-infected and control pigs to examine the effect of *M. suis* on the ECs. Blood vessels and parenchyma were immediately fixed in 4 % phosphate-buffered formaldehyde (FA) for 24 h. Other tissues (lymph nodes, liver, kidney, gut, pancreas, and spleen) were immediately fixed in methanol/glacial acid (2:1 for 24 h) or in Bouin's fluid, and then embedded in paraffin according to standard procedures.

Light microscopy and scanning electron microscopy of aortic vessels

FA-fixed blood vessels and parenchyma were post-fixed in 2.5 % phosphate-buffered glutaraldehyde (GA) for 24 h and then stored in 0.1 M cacodylate buffer until further processing. Strips 2 cm in length (long axis) of the fixed blood vessels were cut in half for macroscopic documentation under a Leica Z16 APO light microscope (Leica Microsystems, Heerbrugg, Switzerland). After microscopy, tissues were post-fixed for 1 h at RT in 1 % osmium tetroxide (Fluka Chemie, Buchs, Switzerland) in 0.1 M cacodylate buffer, dehydrated through a graded ethanol series, and then subjected to critical

point drying (BAL-TEC CPD 030, Critical Point Dryer, Balzers, Liechtenstein). Finally, the samples were sputter-coated with 12 nm of platinum using the BAL-TEC MED 020 coating system, mounted on an aluminum stub, and then analyzed on a Zeiss Supra 50 VP (Oberkochen, Germany) scanning electron microscope.

Immunohistochemical staining of aortic vessels and parenchyma

For immunohistochemistry, the fixed and paraffin embedded tissues were cut into sections (5 µm thick) and mounted on Superfrost® glass slides. After deparaffinization and antigen retrieval in a microwave oven in 10 mM sodium citrate buffer at pH 6.0 (3 × 5 min at 700 W), sections were subjected to immunohistochemistry using an *M. suis*-specific rabbit polyclonal antibody directed against α-enolase (1:200 in PBS) [28]. Briefly, sections were treated with 1 % hydrogen peroxide in double-distilled water for 10 min at RT to block endogenous peroxidase activity followed by 10 % normal goat serum (Dako, Hamburg, Germany) for 30 min at RT to prevent non-specific protein binding. This was followed by incubation with the primary antibody at 5 °C overnight in a humid chamber. The next day, sections were incubated with biotinylated goat anti-rabbit IgG (1:100 in PBS; Dako) for 30 min at RT. Antibodies were detected using a Strept-ABC kit (Dako) according to manufacturer's instructions. Each incubation step was followed by 3 × 5-min rinses with PBS. The reaction product was visualized with 3,3'-diaminobenzidine-hydrogen-peroxide reagent (DAB) (Biotrend Chemicals, Köln, Germany). Finally, sections were counterstained with Mayer's hematoxylin, dehydrated, cleared with xylene, and then mounted in Entellan (Merck, Darmstadt, Germany). The samples were analyzed using a brightfield light microscope (DMRBE, Leica, Bensheim, Germany) equipped with a video camera (ProgRes, Kontron Instruments, Watford, UK). Immunohistochemical controls were performed by (1) replacing the primary antibody with non-immune serum, (2) omitting the secondary antibody, and finally (3) incubation with DAB solution alone to ensure specificity of staining.

Transmission electron microscopy of aortic vessels and parenchyma

Formaldehyde-fixed blood vessels and parenchyma were cut into 1–2 mm segments and post-fixed in 2.5 % phosphate buffered GA solution for 24 h. Samples were washed in 0.05 M cacodylate buffer and stored at 4 °C until further processing. Following post fixation in 2 % osmium tetroxide in cacodylate buffer, sections were washed once in 0.05 M cacodylate buffer. Samples were dehydrated in increasing concentrations of ethanol (70–100 %) and then infiltrated by increasing concentrations (33 and 50 %) of Epon 812 (Fluka Chemie) in ethanol (100%) for 1 h each. After incubation in a mixture of 75% Epon and 25% ethanol overnight, samples were infiltrated with fresh pure Epon for 2-3 h. Specimens were embedded in fresh Epon in flat silicone rubber molds and hardened for 12 h at 60 °C. After preparation of ultrathin sections using an Ultracut E microtome (Reichert-Jung, Vienna, Austria), sections were placed on copper grids (Plano GmbH, Wetzlar, Germany), contrasted consecutively with 4% uranyl acetate (Fluka Chemie) and lead citrate as described previously (Reynold 1963), and then analyzed with a Philips CM100 transmission electron microscope equipped with a Gatan Orius CCD camera (Gatan, Munich, Germany).

Immunogold staining of *M. suis* in aortic tissue

FA-fixed blood vessels were cut into 5–8 mm segments, equilibrated in 30 % sucrose and then embedded in tissue-Tek (Sakura Finetek, Staufen, Germany) at -40 °C. Horizontal segments (40 µm thick) were sectioned using a Kryostat HYRAX C 60 (Zeiss, Jena, Germany) to view the subcellular matrix underneath the endothelium. Sections were permeabilized with 0.2 % Triton X-100 (Sigma-Aldrich, Buchs, Switzerland) for 1 h at RT and then incubated for 24 h with a monoclonal antibody against the *M. suis* surface protein, MSG1 (10 µg/mL). Sections were then washed three times for 20 min each with PBS and then incubated with 1.2 nm colloidal gold particles conjugated to goat anti-mouse IgG (1:1000) [29] for 1 h at RT. Sections were washed three times for 15 min each with PBS and then post-fixed for 30 minutes in 2.5 % GA. After GA fixation, sections were washed twice with distilled water for 20 min. Silver enhancement was performed using the silver enhancement kit (BBI International, Cardiff, United Kingdom). After post-fixation with 2 % osmium tetroxide in PBS, sections were prepared for EM as described for aortic vessels and parenchyma.

***In vitro* adhesion of *M. suis* to PAECs**

PAECs on cover slips were incubated with purified *M. suis* (1×10^4 *M. suis*/mL; 100 µL/well) or the negative control purification preparation (100 µL/well) for different time periods (5 min to 5 days). After washing twice with PBS, cells were fixed in 2.5 % phosphate buffered FA for 2 h at 4 °C. After washing again with PBS, PAECs were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 2 min at RT. Cells were washed with PBS and non-reacted aldehydes were blocked with 0.1 M glycine (Carl Roth, Karlsruhe, Germany) in PBS for 20 min. Non-specific binding of antibodies was reduced by incubation of samples in blocking buffer (3 % BSA in PBS) for 30 min. Adhesion of *M. suis* to PAECs was visualized by staining with rabbit anti-HspA1 serum (1:100) [30] for 1 h followed by TRITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich) for 1 h. Filamentous actin in the PAECs was visualized using FITC-phalloidin (1:80 in PBS; Invitrogen, Basel, Switzerland) and the nucleus was counterstained with DAPI (250 ng/mL). The samples were analyzed on a Leica SP2 confocal laser scanning microscope (CLSM, Leica Microsystems).

FACS analysis of adhesion molecule expression upon interaction with *M. suis*

To analyze the activation of endothelial cells by *M. suis*, PEDSV.15 cells at 60 % confluence were incubated in duplicate with purified *M. suis* (1×10^4 cells /mL; 0.5 mL in PBS) or the negative control purification preparation. Cells were harvested and fixed in 2.5 % FA after 2 h and 4 h for the analysis of E (CD62E)- and P (CD62P)-selectin expression, respectively, and after 24 h, for the analysis of intercellular adhesion molecule 1 (ICAM-1; CD54) and platelet endothelial cell adhesion molecule 1 (PECAM-1; CD31) expression [31-33]. These time points corresponded to the maximum up-regulation of activation markers determined with the positive control, i.e. tumor necrosis factor a (TNF-α) and lipopolysaccharide (LPS; Sigma). Fixed cells were incubated with 100 µL of blocking buffer (10% BSA in PBS) for 30 min at RT, followed by specific antibodies directed against ICAM-1 (clone 19C7; a kind gift from D. Haskard, Imperial College London, UK), PECAM-1 (LCI-4; AbD Serotec, Oxford, UK), P-selectin (clone 12C5; D. Haskard) and E-selectin (clone 1.2B6; AbD Serotec) as previously described [34, 35]. FITC-conjugated goat anti-mouse IgG (1:1000; Sigma-Aldrich) was

used as a secondary antibody. Samples were analyzed using a FACSCanto II cell sorting system (BD Biosciences, Allschwil, Switzerland). Experiments were performed three times.

SPSS statistical software was used to test differences in the expression of cell surface markers between groups. Differences were calculated using the unpaired Wilcoxon test and a P value ≤ 0.05 was considered statistically significant.

Results

Endothelial cell alteration and activation in *M. suis*-infected pigs

Microscopic analysis of the vascular endothelium of *M. suis*-infected pigs revealed marked alterations of the luminal surface of the abdominal aortic vessel, which appeared rough and covered in reddish spots (Appendix IA). These red dots indicated aggregated blood cells and perforation of the vessel walls. By comparison, the endothelial surface of the abdominal aortic vessel in healthy pigs exhibited a smooth and even surface structure (Appendix IB). Histological and immunohistochemical analysis of the organs of *M. suis*-infected pigs (gut tissue, heart, lung, liver, lymph nodes, and spleen) revealed clear evidence of hemorrhage in the tissues and massive alterations in tissue structure, particularly in the vascular tissue. Infection with *M. suis* also resulted in injury to the ECs lining the post-capillary venules and small veins. As shown in Figure 1, early signs of degeneration, i.e. massive cellular protrusions into the vessel lumen, were accompanied by acute cytoplasmic swelling. Subsequently, desquamation, necrosis and detachment of ECs from the underlying basement membrane were observed, resulting in progressive cellular denudation of the luminal surface. Numerous *M. suis* cells could be detected within the detached endothelium (Figure 1).

Scanning electron microscopy (SEM) of the aortic vessel of *M. suis*-infected pigs revealed areas of endothelial denudation leading to an exposed sub-endothelial matrix. In the endothelial gaps, there were large numbers of leukocytes embedded within a fibrin network. The remaining ECs were covered with numerous microvilli, indicating an inflammatory response (Figure 2A and 2B). Areas with adherent blood cells, which had appeared as red dots by light microscopy, where identified as arterial thrombi by SEM. These arterial thrombi were characterized by the presence of RBCs and leukocytes embedded in a network of fibrin fibers (Figure 2C).

SEM also revealed *M. suis* aggregates of approximately 5–50 μm in diameter attached to the surface of the vessel walls. These micro-colonies were characterized by round or elongated cross-linked cells of 200–400 nm in diameter embedded in a three-dimensional granular matrix (Figure 2D). In addition, smaller coccoids (less than 100 nm in size) localized to the surface of the larger bacterial cells (Figure 2E). In some cases, *M. suis* cells on RBCs were interconnected via small fibrils (Figure 2F). In contrast to aortic vessels from *M. suis*-infected pigs, the aortic vessels of healthy pigs showed no evidence of endothelial cell damage. Confluent layers of spindle-shaped ECs (5–8 μm in width and 10–25 μm in length) arranged in parallel and with no attached blood cells or bacteria were observed in non-infected pigs (Figure 2G and 2H).

Ultrathin sections of the abdominal aorta were further analyzed for EC activation and detachment by transmission electron microscopy (TEM). Microvilli formation was evident on the ECs of the abdominal aorta of *M. suis*-infected pigs (Figure 3A). In some ultrathin sections, no ECs were detected at all, a finding consistent with the SEM analysis of the aorta of the same pig (Figure 3B). *M. suis* was detected in the subcellular aortic tissue following staining with an anti-MSG1 monoclonal antibody and subsequent nanogold silver intensification (Figure 3C, D). *M. suis* cells appeared to be attached to subcellular matrix structures resembling collagen. As with the results of SEM, the vascular wall of healthy control pigs was characterized by a continuous layer of ECs with no alterations in ultrastructure (Figure 3E).

Intravascular coagulation and morphological changes in the parenchyma in *M. suis*-infected pigs

To further examine *M. suis*-induced morphological changes, endothelial morphology and intravascular coagulation in the organ capillaries of the liver and heart parenchyma were analyzed by TEM.

The ECs of the heart capillaries of *M. suis*-infected pigs were irregular in shape with localized swelling and finger-like pseudopodia (Figure 4A, B). *M. suis*-infected RBCs were adherent to the endothelium and surrounded by endothelial pseudopodia.

Morphological changes in the heart parenchyma of infected pigs were also observed. In cardiac muscle cells (Figure 4A), myofibrils were disarranged and the number of fibrils was reduced. Mitochondria were markedly reduced in number and appeared to be randomly distributed in the heart tissue. Sarcomere atrophy was evident and the structures inconsistent in many parts of the tissue (Figure 4A).

In control pigs, by comparison, there was a regular pattern of ECs lining the cardiac vessel wall. No RBCs were attached to the vessel wall and no *M. suis* particles were detected (Figure 4C). The morphology of the cardiac muscle of control pigs was normal, with alternating I and A bands. Sarcomeres were arranged in parallel and myofibrils were separated by the normal striped arrangement of mitochondria.

Intravascular coagulation was detected in the microvascular channels (sinusoids) of the liver of *M. suis*-infected pigs. Aggregated RBCs attached to the vascular wall appeared to block the sinusoids (Figure 4D, E). In contrast, plasma in the sinusoids of control pigs had a homogenous appearance and RBCs were distinct and separated from the vessel wall (Figure 4F). Liver cells (hepatocytes) from *M. suis*-positive pigs (Figure 4D, E) and control pigs (Figure 4F) had a normal polygonal structure and showed no signs of morphological changes. Bile canaliculi, the thin tubes that collect bile secreted by hepatocytes, were also displayed.

M. suis **interaction with and activation of porcine aortic endothelial cells** ***in vitro***

To investigate the interaction of *M. suis* with ECs *in vitro,* PAECs were infected with purified *M. suis* and then analyzed by confocal laser scanning microscopy following immunohistochemical staining to differentiate the cytoskeleton, cell nucleus, and *M. suis. M. suis* cells were detected as aggregates on the surface of PAECs as early as 90 min post-infection. Actin condensation was observed at the sites of bacterial cell attachment (Figure 5A–C) and always co-localized to sites of bacterial aggregates. Propagation of *M. suis* on the surface of PAECs was not observed over the course of this experiment. As a control, no *M. suis* cells were detected on PAECs incubated with the negative control preparation (Figure 5D).

To assess whether EC activation occurred as a result of the direct interaction with *M. suis,* PEDSV.15 cells were infected with purified *M. suis*. Expression of different cell adhesion molecules (CAMs), i.e. ICAM-1 (CD54), PECAM-1 (CD31), P-selectin (CD62P) and E-selectin (CD62E) was used as activation marker. The CAMs expression levels (number of CAMs expressing PEDSV.15 cells) were determined using FACS analysis and CAM-specific monoclonal antibodies. Responsiveness of the PEDSV.15 cells was proven by the induction of CAMs upregulation after TNF-α and LPS stimulation.

Overall, there was a significant increase in CAMs expressing PEDSV.15 cells upon infection with *M. suis* ($P \leq 0{,}05$). As shown in Figure 6 *M. suis*-stimulated PEDSV-15 showed a significant increase in CAM cell-surface expression compared with that in the negative control bacterial preparation *M. suis*-incubated PEDSV-15 expressed on average 1.9-fold more ICAM-1 (CD54, $P = 0.028$), 2.6-fold more E-selectin (CD62E, $P = 0.043$), 7.5-fold more P-selectin (CD62P, $P = 0.028$) and 1.9-fold more PECAM-1 (CD31, $P = 0.043$) compared with negative control bacterial preparation.

Discussion

Hemolytic anemia as a result of acute *M. suis* infection is accompanied by coagulopathy, vascular thrombosis, and hemorrhaging of the skin and organs; symptoms that are indicative of the involvement of vascular ECs in the pathogenesis of acute *M. suis* infection [9, 11, 12]. However, the involvement of ECs in *M. suis* pathogenesis has received little attention to date because consumption coagulopathy is considered to be the primary cause of hemorrhagic diathesis [12]. Our own recent clinical observations of acute IAP with a fatal outcome [1] led to the hypothesis that interactions with ECs contribute to IAP pathogenesis. Therefore, the aim of the present study was to investigate the interaction between *M. suis* and ECs, and the resulting endothelial disruption and damage to the vasculature.

SEM and TEM analyses clearly demonstrated marked abnormalities in the endothelium of blood vessels in *M. suis*-infected pigs. Blood vessels exhibited detached ECs over a considerable portion of the endothelium. In some ultrathin sections, EC were completely missing and the subcellular matrix was exposed. This likely represents the mechanism by which *M. suis* gained access to the

sub-endothelial matrix, where it was detected by TEM. However, the extent of endothelial denudation made the microscopic detection of intracellular *M. suis* in ECs unfeasible. An analogy can be made with certain *Bartonellae* species, which are also undetectable intracellularly in ECs, even though pathogenesis induced by these species was shown to involve ECs [36]. Additionally, the abnormalities reported herein are typical signs of activated ECs seen in several other diseases, i.e. sickle cell anemia [37, 38], and are highly indicative of dysfunctional activation of the endothelium in IAP.

The formation of microvilli-like membranes on ECs was observed in *M. suis*-infected animals, which is also characteristic of an activated phenotype, and could serve to increase the frequency of adhesion of *M. suis*-infected RBCs, as observed in micro vessels from patients infected with *P. falciparum* [39]. The increased adhesion of *M. suis*-infected RBCs to the endothelium might be further enhanced by *M. suis*-induced RBC changes, some of which could trigger a variety of pathologies such as vasoocclusion, endothelial activation, and initiation of the coagulation cascade [40]. The extent of RBC adhesion seen in the liver sinusoids, as well as in heart capillaries, might be a result of a combination of activated ECs with microvilli, altered RBC membranes, and an increased adhesiveness caused by pseudopodia. Similar pathologic patterns are observed in *P. falciparum* infections [41].

Numerous arterial thrombi, composed of leukocytes, RBCs, platelets and a network of fibrin fibers, were attached to ECs next to sites of *M. suis*-induced endothelial injury. This fibrin network acts as a provisional matrix during inflammation and wound healing [42]. Fibrin and fibrinogen metabolites bind to activated platelets and also to vascular EC, thereby activating both cell types [42, 43]. Activated ECs facilitate the translocation of phagocytes into the injured tissue to eliminate bacteria via phagocytosis [44]. Furthermore, in diabetes mellitus and sickle cell anemia, fibrinogen enhances adhesion of pathological RBCs to ECs, and this is associated with a high incidence of thrombosis [45, 46].

The detection of *M. suis* in direct contact with ECs and attached to the endothelium is an entirely new finding in HM research. To date, HM has been reported to attach and invade a unique host cell, the RBC [1-3]. In the current study, *M. suis* was attached to ECs either as a single cell or as microcolonies ranging in diameter from 30–50 µm. This direct interaction of *M. suis* with ECs could trigger the EC destruction and exposure of the subendothelial matrix observed in the *M. suis*-infected pigs. This

would be consistent with other pathogenic *Mycoplasma* species, such as the large colony strains of *M. mycoides* subsp. *mycoides*, which are able to adhere to and destroy caprine ECs, thereby exposing subendothelial collagen and causing vasculitis and coagulation disturbances [18, 20].

The *M. suis* biofilm-like structures we observed were homogenous aggregates of cross-linked 200-400 nm *M. suis* cells that formed a three-dimensional network. Occasionally, microcolonies appeared more compact and the bacteria were embedded in a granular matrix. *M. suis* microcolonies attached to the vascular wall and resembling biofilms have been described for other mycoplasmas [47]. Within these microcolonies, smaller coccoid cells less than 100 nm in diameter were observed on the surface of the larger cells. These *M. suis* cells probably originated from a budding-like replication mechanism, similar to that proposed for HM, as well as for other mycoplasmas, and they resembled propagating *M. suis* cells on RBC [1, 48, 49]. These results strongly suggested that *M. suis* is capable of propagating on ECs.

One of the more novel and more interesting findings of this study was the formation of small fibrils interconnecting several *M. suis* cells on the surface of RBCs and attached to the vascular wall. *M. suis* adhere to host RBCs via fibrils [49, 50]. In the current study, the interconnected *M. suis* cells were observed only on the surface of RBCs attached to the endothelium. This type of specialized cell-to-cell communication may be necessary for the development of an endothelial biofilm. The gram-negative *Myxococcus xanthus* is able to form extensive fibrillar interconnections, which are necessary for their characteristic social behavior [51]. Further studies are needed to better characterize the composition and function of *M. suis* fibrils.

In vitro analysis using porcine endothelial cells (PAEC and PEDSV.15 cells) provided further insight into the mechanism of interaction between *M. suis* and ECs. A considerable number of *M. suis* cells were attached to the PAEC surface, and actin condensation was evident at the attachment sites. Indications for an interaction between *M. suis* and porcine actin have been found in a previous study since *M. suis*-induced autoreactive IgG antibodies also bind to actin [8, 52]. In the present study, we found clear evidence that *M. suis* interacts with cytoskeletal actin of endothelial cells leading to cytoskeletal rearrangements in the EC, which might allow internalization of *M. suis*. Various invasive bacteria, such as *Yersinia enterocolitica* and *Listeria monocytogenes,* trigger rearrangement of the

actin cytoskeleton to facilitate their uptake into eukaryotic cells [53, 54]. Analysis of the *M. suis* genome revealed that, similar to other *Mycoplasma* species, *M. suis* lack any of the genes required for *de novo* nucleotide biosynthesis [55]. Thus, establishing itself in a new niche in a nucleated cell would be of critical importance for *M. suis* in terms of acquiring a source of nucleotides for proliferation. Despite demonstrating a clear and close interaction between *M. suis* and ECs, definitive proof of the intracellular localization of *M. suis* in ECs *in vivo* could not be demonstrated due to the extensive cytopathological effects of *M. suis* (data not shown). Damaged ECs most likely detached from the endothelium and, thus, were not available for microscopic analysis of intracellular *M. suis*. Additional studies of sections from infected blood vessels at an early phase of infection may provide the necessary evidence to support this hypothesis.

The activation of ECs by *M. suis* was demonstrated *in vitro* by the significant increase of ICAM-1, PECAM, E-selectin and P-selectin expressing cells, all of which are important markers of EC activation. Endothelial E-selectin and P-selectin are up-regulated by inflammation and mediate leukocyte capture and rolling on the endothelium [56]. The up-regulation of E-selectin is associated with organ dysfunction and septic shock [57], both of which are seen in acute IAP. The time point of a maximum upregulation of activation markers by LPS and TNF-α might not represent the time point of a maximum upregulation by *M. suis*. This could explain the relatively smaller but significant difference between *M. suis* and negative control in case of E-selectin and PECAM-1. ICAM-1 is involved in leukocyte rolling and arrest on endothelial cells [56], as well as the movement of neutrophils and monocytes on the endothelium [58]. Recently, ICAM-1 was identified as the receptor for rhinovirus [59]. Furthermore, PECAM-1, which is expressed on the surface of ECs and most leukocytes, was up-regulated upon *in vitro* stimulation with *M. suis* cells. PECAM-1 is involved in the removal of apoptotic neutrophils from the body and makes up a large proportion of the endothelial cell intercellular junction [60, 61], where it mediates transendothelial migration via homotypic binding to PECAM-1 on leukocytes [62]. In summary, these *in vitro* findings, including the up-regulation of endothelial adhesion receptors, together with the observed structural changes of the endothelial layer *in vivo*, i.e. microvilli- and gap-formation, demonstrate that *M. suis* infection results in activation of EC.

Another of the more intriguing findings of the current study was the observation of cardiac muscle damage with disorganized and damaged and/or destroyed cardiac muscles cells. These effects could be explained by RBC aggregation in the blood vessels of *M. suis*-infected pigs with subsequent occlusion of capillaries leading to ischemia (interruption of the blood supply). This would be consistent with the fact that some cases of acute *M. suis* infection in pigs result in death within a few days [1].

In conclusion, we report several novel findings of infection with HM leading to widespread endothelial damage, RBC adhesion to the endothelium, and vascular occlusion. These vascular alterations lead to the development of hemorrhage and organ failure. To our knowledge, this is the first demonstration that HM interacts with host cells other than RBCs. In addition, the ability of *M. suis* to form biofilm-like microcolonies on the endothelium, which may protect the organism from antimicrobial agents and host immune factors, may contribute to the persistence of HM infections.

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Figures

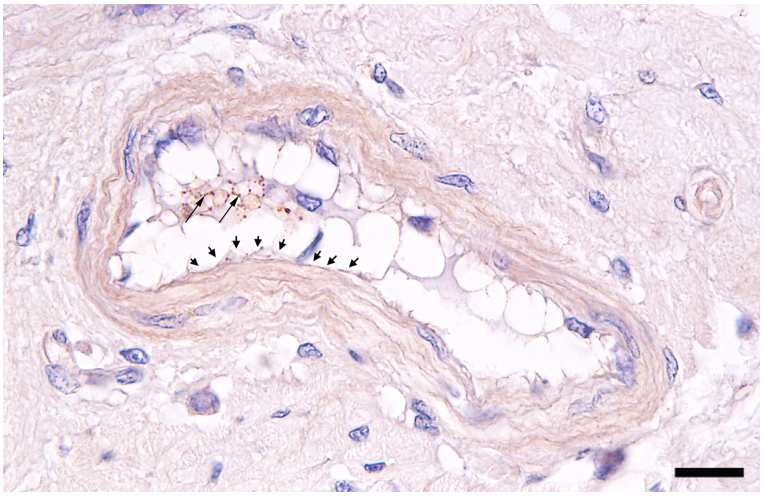


Figure 1: Degeneration of capillary endothelial cells in *M. suis*-infected pigs.

Representative small intestinal venule of an *M. suis*-infected pig (histological analysis). Massive cytoplasmic swelling of ECs, accompanied by progressive cellular necrosis and detachment of ECs from the underlying basement membrane, is evident. Arrowheads indicate areas of cellular denudation of the luminal surface; small arrows indicate immunoreactive *M. suis* cells. Scale bar = 20 μm.

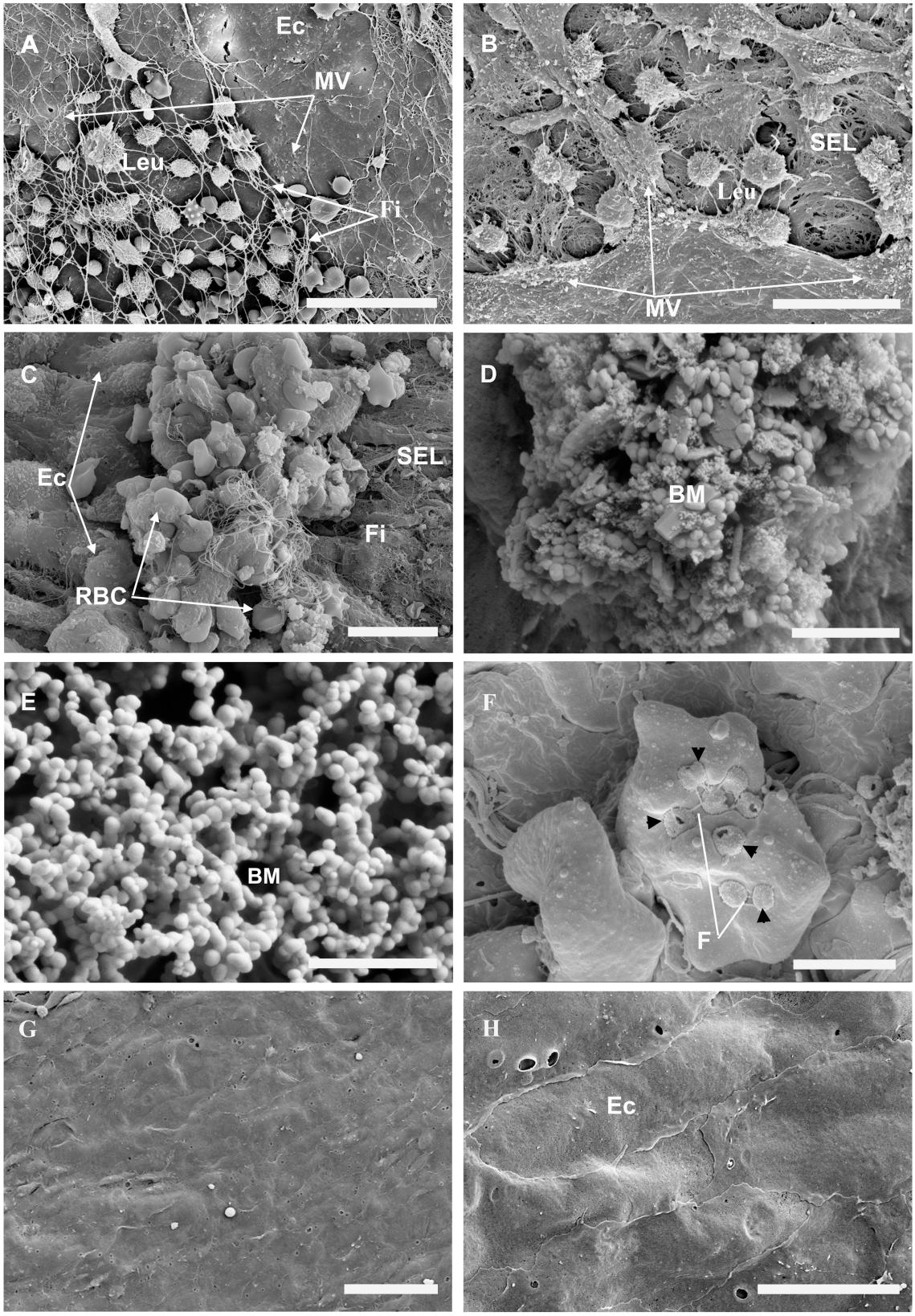


Figure 2: Endothelial denudation and microvilli and microcolony formation due to *M. suis* infection.

A. Aortic vessel of an infected pig with adherent blood cells and fibrin fibers attached to ECs. Scale bar = 20 μm; B. Aortic vessel from an infected pig showing activated ECs (microvilli formation) and

detached ECs exposing the subendothelial layer (SEL). Leukocytes are attached to the ECs and the subcellular matrix. Scale bar = 10 μm; C. Vascular wall covered with blood clots. Scale bar = 10 μm; D. Bacterial microcolonies (BMs) attached to the vascular wall. Scale bar = 2 μm; E. *M. suis* coccoids of less than 100 nm. Scale bar = 2 μm; F. *M. suis* cells attached to RBCs and interconnected via small fibrils. Scale bar = 2 μm; G. Luminal surface of an aortic vessel from a healthy pig showing a confluent layer of ECs (overview). Scale bar = 40 μm; H. Aortic vessel from a healthy pig showing a confluent layer of ECs. Scale bar = 10 μm. Abbreviations: BM, bacterial microcolony; EC, endothelial cell; Fi, fibrinogen fibers; Leu, leukocytes; MV, microvilli; RBCs, red blood cells; F, small fibrils; SEL, subendothelial layer; and VW, vessel wall.

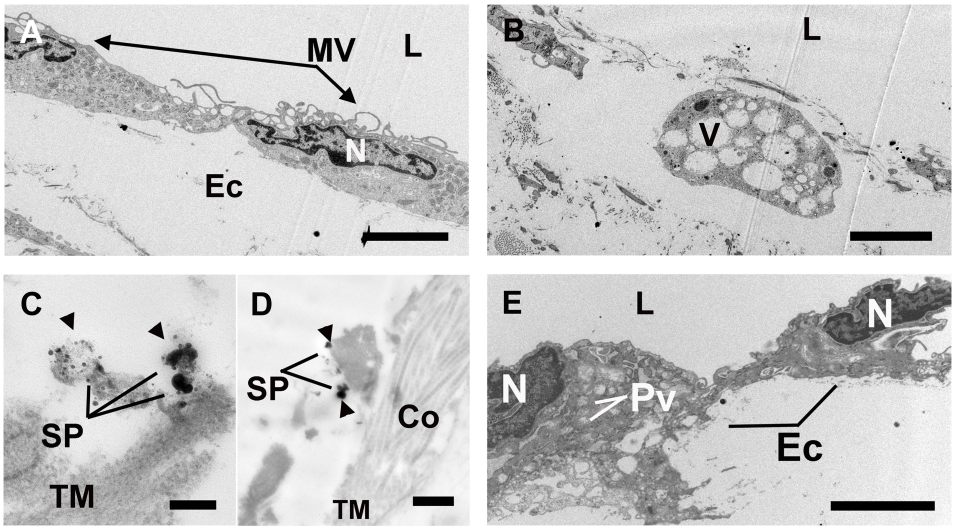


Figure 3: *M. suis* infection induces endothelial cell detachment and activation.

Transmission electron micrographs of aortic sections. A. ECs (black arrows) of pigs infected with *M. suis* (black arrowheads) are characterized by extensive microvilli (MV) formation. Scale bar = 4 μm; B. Absence of ECs/EC detachment and remaining vacuolar cells. Scale bar = 4 μm; C, D. *M. suis* was detected in the aortic sub-cellular tissue by nanogold silver intensification staining. Scale bar = 0.2 μm (C); Scale bar = 0.5 μm (D); E. Endothelial cells (ECs) of uninfected control pigs showing normal ultrastructure. Scale bar = 4 μm. Abbreviations: Co, collagen; L, lumen; PV, plasmalemma vesicle; N, nucleus; SP, silver precipitates; RM, tunica media; and VC, vacuolar cell.

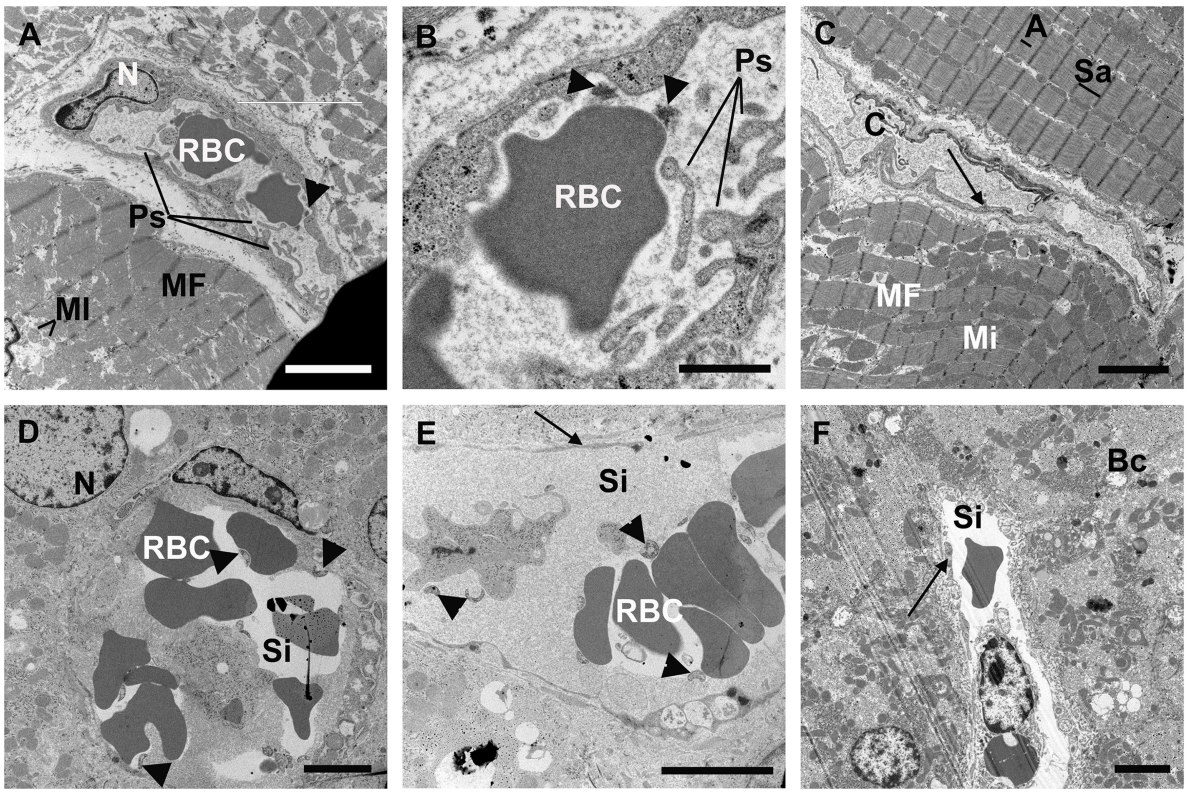


Figure 4: Intravascular coagulation and morphological changes in *M. suis* infected pigs.

Transmission electron micrographs of porcine liver and heart preparations. A, B. Heart biopsies from an *M. suis*-infected pig with unorganized myofibrils (MF). *M. suis* cells are indicated by a black arrowhead and thin endothelial cells of capillaries (c) and sinusoids (Si) with a black arrow. Scale bar = 4 μm (A); scale bar = 4 μm (B); C. Heart biopsy from an uninfected control pig. Myofibrils exhibit the typical banding pattern with alternating A and I bands. Scale bar = 4 μm. D, E. Liver preparations from an *M. suis*-infected pig with red blood cell (RBC) aggregates adherent to endothelial cells (ECs). Scale bar = 4 μm (D); scale bar = 1 μm (E); F. Liver preparation from an uninfected control pig. Scale bar = 4 μm. Abbreviations: Mi, mitochondria; N, nucleus; Ps, pseudopodia; RBC, red blood cell; ER, endoplasmic reticulum; V, intracellular vacuoles; and Bc, bile canaliculi.

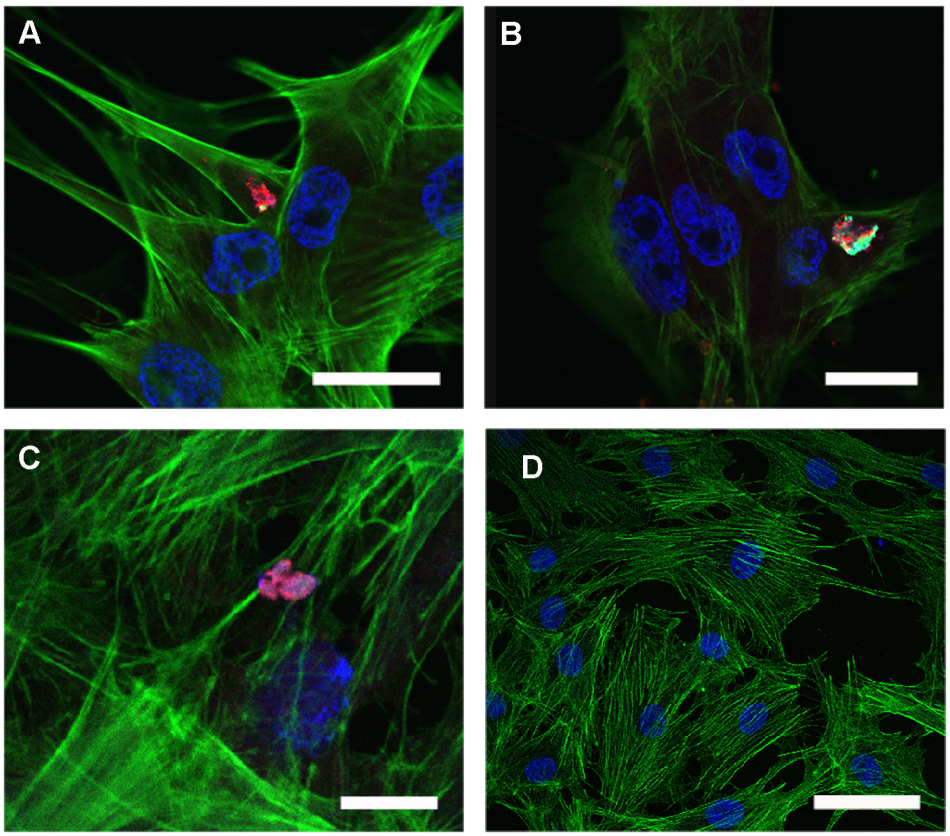


Figure 5: Interaction of *M. suis* with porcine endothelial cells induces actin condensation *in vitro*.

Confocal laser scanning micrographs of PAECs incubated with *M. suis* (1×10^4 cells/mL). Cytoskeletal actin was stained with FITC-phalloidin (green); *M. suis* cells were stained with anti-HspA1 antiserum and TRITC-labeled secondary antibodies (red). Nuclei and bacteria were counterstained with DAPI (blue). The image represents an overlay of all fluorescent channels. (A) 90 min post-infection (pi); scale bar = 40 μm; (B) 6 hours pi; scale bar = 20 μm; (C) 4 days pi; scale bar = 10 μm; (D) Pre-infection; scale bar = 50 μm.

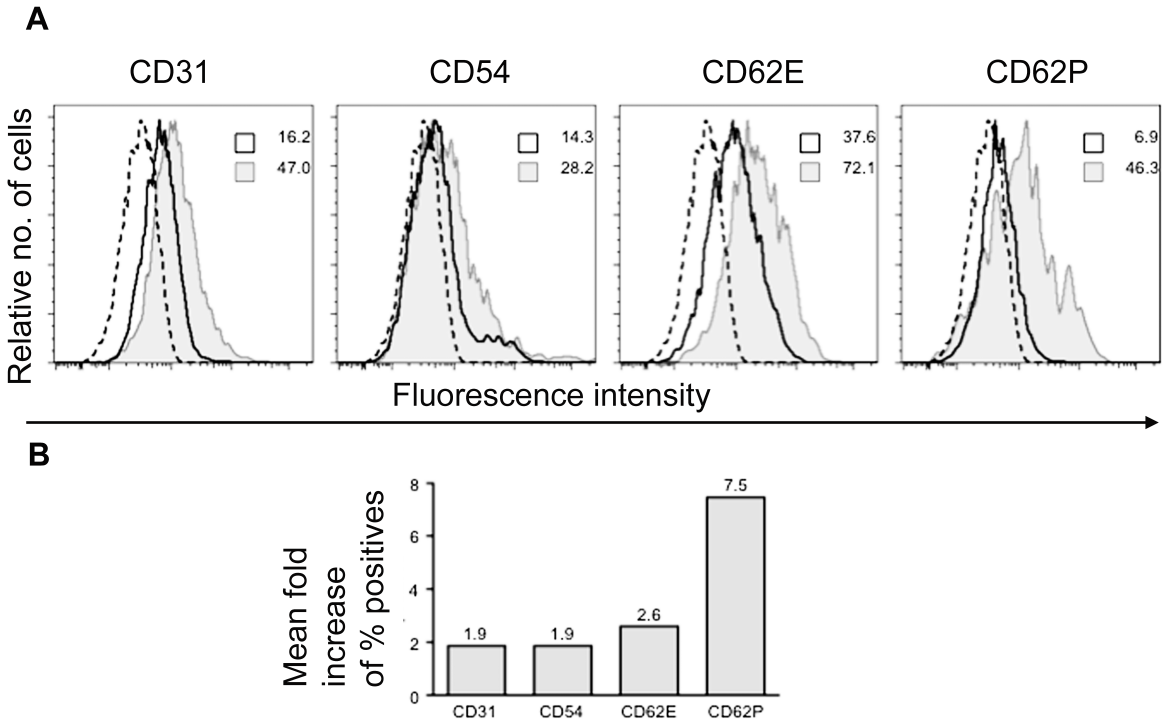


Figure 6: CAM expression in *M. suis* infected endothelial cells.

The expression profiles of adhesion molecules on endothelial cells were analyzed using a FACSCanto II cell sorting system. A. Dashed lines represent the isotype control; solid lines represent cells incubated with the negative control bacterial preparation; grey histograms represent PEDSV.15 cells incubated with *M. suis* (1×10^4 cells/mL). B. Percent increase in expression ICAM-1 (CD54), PECAM-1 (CD31), E (CD62E)- and P (CD62P)-selectin in ECs infected with *M. suis*.

5 Milestones and Outlook

Chronic course of *M. suis* infections result from the ability of *M. suis* to make use of different strategies that enable agent life-long persistence. Several processes contributing to *M. suis* persistence were evidenced in the present thesis and are summarized in figure 9: (1) *in vivo* and *in vitro* impairment of host immune response upon *M. suis*-host interaction was documented; (2) The formation of *M. suis* microcolonies, that were found attached to the endothelium as well as PBMCs, also contributes to the persistence of the pathogen. Targeting components of the immune system enables *M. suis* to evade and modulate this system leading to a productive niche for the persistent agent propagation and to autoreactive processes.

In addition, dysfunctional activation of endothelium induced by *M. suis* infection resulted in the immunopathological features e.g. hemorrhagic diathesis, coagulopathy, tissue damage, and organ failure.

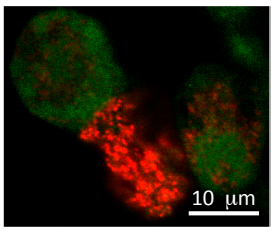
- The present study provides for the first time evidence of *M. suis* interaction with cell types other than RBCs. *M. suis* microcolonies attach to endothelial cells (ECs) and PBMCs, thereby inducing activation of ECs and clotting several PBMCs together. *M. suis* single cells and cell clusters were found localized inside PBMCs. Since the *M. suis* positive PBMCs subtype is not identified, it is not clear whether *M. suis* internalization is an active bacterial process or rather attributed to phagocytosis. Further approaches of our research group deal with the identification of the type of internalization of *M. suis* by PBMCs.

- *M. suis* interacts with PBMCs and induces premature activation of T-regulatory cells. T-regulatory cells inhibit the proliferation of T-helper cells via production of IL-10. The initial *in vitro* activation of Th1 immune response is downregulated early before an *M. suis* specific Th1 immune response establishes. The down regulation of IL-12 production correlates negatively with the clearance of intracellular *M. suis,* thus enabling survival of intracellular *M. suis*. In parallel B-lymphocytes are activated polyclonally upon *M. suis* encounter in

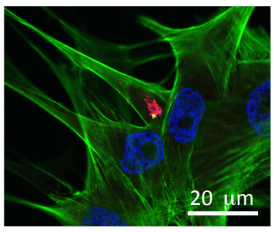
T-helper cell independent manner. The polyclonally activated B-lymphocytes produce autoreactive antibodies, those are responsible for the *M. suis* induced autoimmunity in IAP. *M. suis* induced upregulation of proinflammatory cytokines e.g. IL-1, IL-6, and TNF-α most probably contribute to the immunopahtology in IAP. The upcoming *in vivo* investigations shall confirm the outcome of *in vitro* assays.

- The coagulopathy induced by *M. suis* infection results from the pathological activation of endothelial cells upon interaction with *M. suis*. The majority of PBMCs were trapped in the coagula, a condition that led to leukopenia. The pathological activation of the endothelium and coagulopathy are responsible for the thrombocytopenia, leukopenia, and hemorrhagic diathesis, of which the latter contributes to the spread of the *M. suis* into the surrounding tissue.

A. *M. suis* inside PBMCs and ECs

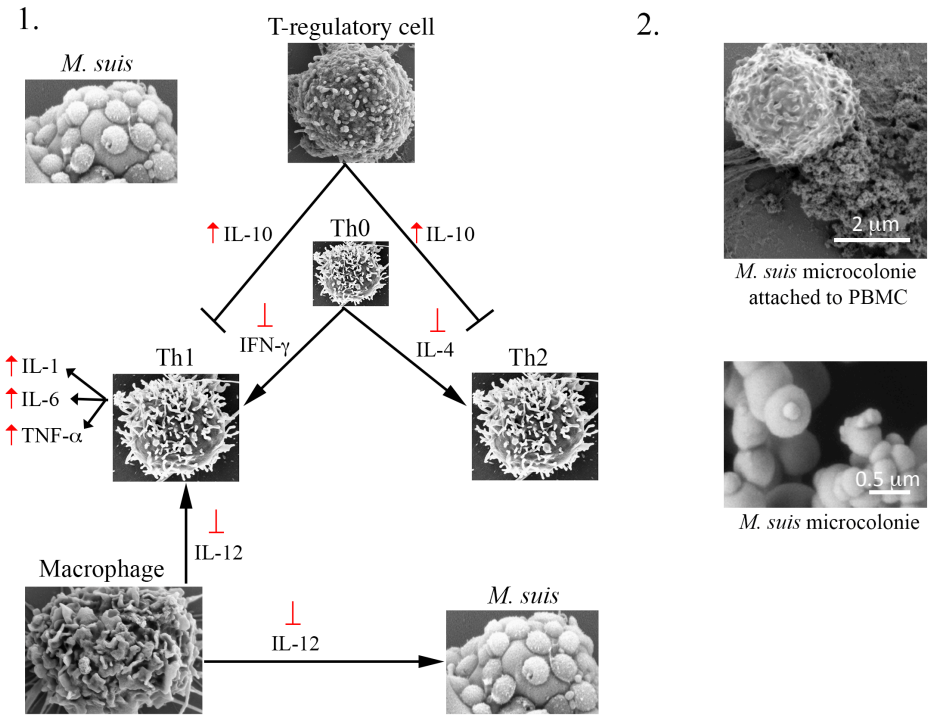


PBMC (green); *M. suis* (red)

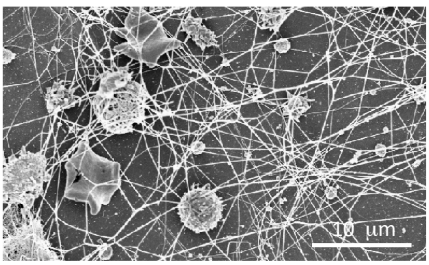


ECs (green); Nucleus (blue); *M. suis* (red)

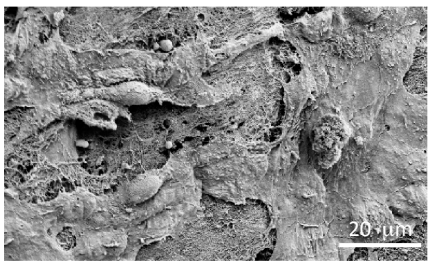
B. *M. suis* persistence via (1) immune modulation and (2) biofilm formation



C. Coagulopathy and dysfunctionally activated endothelium



Blood clot



Damaged endothelium

Figure 9: Milestones of the thesis. A. *M. suis* was detected inside PBMCs and ECs; B. *M. suis* modulates the immune response of the host and forms biofilm-like structures to persist. (1) Components of the immunes system targeted by *M. suis* are shown in red. Interaction with *M. suis* leads to induction of: IL-1, IL-6, IL-10, TNF-α and inhibition of IL-4, IL-12, and IFN−γ; (2) *M. suis* microcolonies resemble biofilm formations; C. Pathological alterations occurred during *M. suis* infection.

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7 CV and list of publications

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Grants

02/2010-02/2011	Financial support by Swiss National Science Foundation (SNSF) Project: *In vitro* and *in vivo* studies on the molecular mechanisms leading to persistence, anemia/circulatory disturbances, and immune modulation in *Mycoplasma suis* infections
07/2012	Travel grant by microbiology and immunology (MIM) PhD program for attending the 19th Congress of the International Organization of Mycoplasmatology (IOM), 17-19 July 2012, in Toulouse (France).

List of publications

Ender M, Burger S, Sokoli A, Zbinden R, Berger-Bächi B, Heusser R, Senn MM, McCallum N. **Variability of *SCCmec* in the Zurich areas.** Eur J Clin Microbiol Infect Dis 2008, (28):647-653.

Schreiner SA, Sokoli A, Felder KM, Wittenbrink MM, Schwarzenbach S, Guhl B, Hoelzle K, Hoelzle LE: **The surface-localised alpha-enolase of *Mycoplasma suis* is an adhesion protein.** Veterinary microbiology 2012, 156(1-2):88-95.

Schreiner SA, Hoelzle K, Hofmann-Lehmann R, Hamburger A, Wittenbrink MM, Kramer MM, Sokoli A, Felder KM, Groebel K, Hoelzle LE: **Nanotransformation of the haemotrophic *Mycoplasma suis* during *in vitro* cultivation attempts using modified cell free Mycoplasma media**. *Veterinary microbiology* 2012.

Sokoli A, Groebel K, Hoelzle K, Amselgruber WM, Mateos JM, Schneider MK, Ziegler U, Felder KM, Hoelzle LE: **Mycoplasma suis infection results endothelial cell damage and activation: new insight into the cell tropism and pathogenicity of hemotrophic mycoplasma**. *Vet Res* 2013, **44**(1):6.

Contribution to international conferences

2008, 19th congress of the International Organisation of Mycoplasmology (IOM) in Toulouse, France.

Poster entitled "***Mycoplasma suis* infection results endothelial cell damage and activation: new insight into the cell tropism and pathogenicity of hemotrophic mycoplasma** ".

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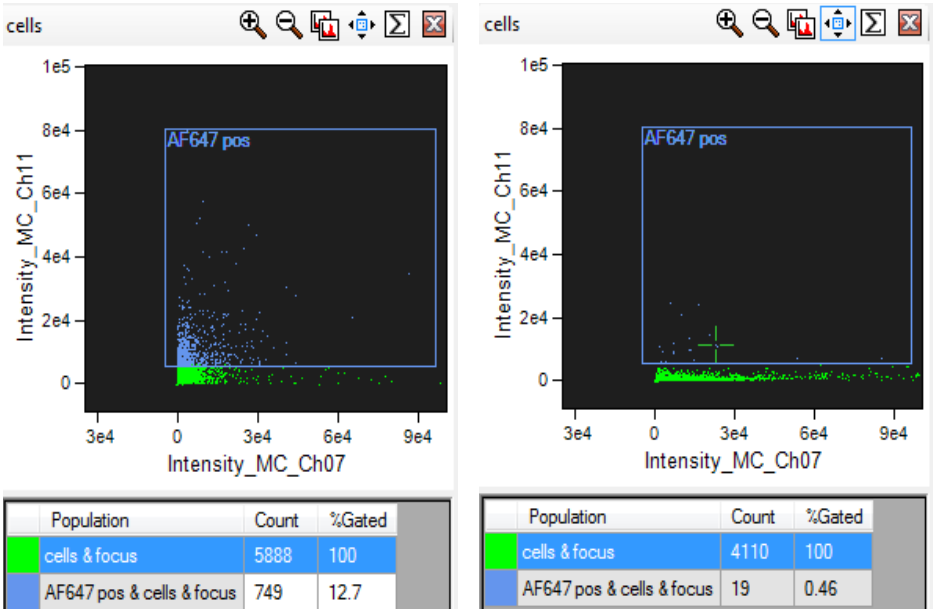
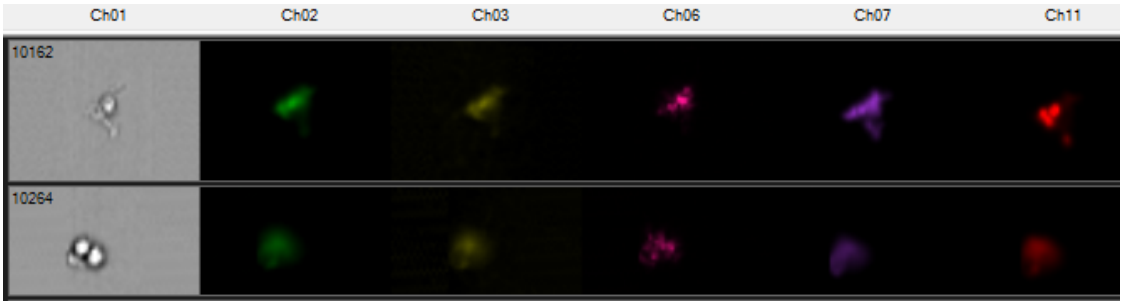
9 Appendix I

A



B.



Appendix I: Quantitative imaging analysis of PBMCs in flow using FlowSight

FlowSight cytometer provides visual verification of the morphology of each cell analyzed and enables gating of cells from debris. A. In samples from *M. suis* infected pig (No 6019) 12.7 % of the PBMCs were *M. suis* positive, whereas PBMCs from *M. suis* negative pig were all negative; B. In chanel (Ch) 01 birghtfield images of 2 *M. suis* positive PBMCs from Pig No 6019 are shown. The analyzed *M. suis* positive PBMCs were whole, intact single (10162) or cluster (10264) of cells.